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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The ability of cells to change their shape and move is critical to many biological processes. Cancer metastasis, the process whereby cancer cells leave a primary tumor and proliferate at distant sites, is highly dependent on cell shape changes and cell motility. The Rho GTPases play major roles in regulating the organization of the actin cytoskeleton and cell motility. Cdc42, one of the Rho GTPases, exerts its biological effects through interaction with downstream effector proteins. We identified a new family of Cdc42 effector proteins called SPECs for Small Protein Effector of Cdc42. The two human members of this family, SPEC1 and SPEC1 contain a centrally located CRIB domain through which they bind Cdc42. Overexpression of SPEC1 and SPEC2 in NIH-3T3 fibroblasts resulted in the formation of plasma membrane blebbing and modified Cdc42-induced biological activities when co-expressed with Cdc42. JNK kinase assays revealed that SPEC1 inhibited Cdc42-induced JNK activation. Northern blot analysis showed that SPEC1 is alternatively spliced and exists in at least four different molecular weight species. Additional Northern blot data using RNA from a panel of breast cancer cell lines suggests that SPEC1 expression may decrease in more metastatically aggressive breast cancer cell lines.

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Introduction

As research in the field of cancer treatment progresses, new therapeutics are continually being developed to fight cancer. Despite these advances, cancer metastasis remains a major obstacle to effective cancer treatment and the major cause of cancer mortality. Cancer metastasis is the process whereby cancer cells leave a primary tumor, enter the circulation, extravasate and proliferate at distant sites (Fidler et al., 1978). At a cellular level, the acquisition of a motile and invasive phenotype requires the abrogation of cell-cell contacts, the remodeling of the extracellular matrix, and changes in cell-matrix interactions, leading ultimately to the reorganization of the actin cytoskeleton and cell motility. A family of proteins called the Rho GTPases play major roles in regulating these very processes, including the regulation of the actin cytoskeleton leading to cell shape changes, the establishment of cell-cell contacts, cell-matrix interactions, and cell polarization (reviewed in Van Aelst et al., 1997). Because of the participation of the Rho GTPases in cellular processes involved in cell motility and migration, it is not unlikely that the aberrant regulation of the Rho GTPase signaling pathways may play critical roles in cancer metastasis. Thus, understanding the proteins that bind and regulate the Rho GTPases is critical to our understanding of cell motility as well as having exciting potential applications for cancer metastasis.

The Rho GTPases belong to the Ras superfamily of small G proteins that function as molecular switches to relay signals downstream of transmembrane receptors. The prototypical members of this family, Rho, Rac, and Cdc42 play a role in cell motility by regulating structures formed by the cortical actin cytoskeleton. In Swiss 3T3 fibroblasts, microinjection of Cdc42 leads to the formation of filpodia, microinjection of Rac induces the formation of lamellipodia, and microinjection of Rho causes the formation of stress fibers (Kozma et al., 1995; Nobes and Hall, 1995). Furthermore, in Swiss 3T3 fibroblasts, these GTPases cross-activate each other in a cascade fashion, where activation of Cdc42 leads to activation of Rac and subsequent activation of Rho (Nobes and Hall, 1995). Thus, Rho GTPases work cooperatively to mediate the process of cell motility (Nobes and Hall 1999). In addition to their effects on the actin cytoskeleton, the Rho GTPases are also implicated in a variety of other signaling pathways, including the assembly of focal adhesions complexes (Nobes and Hall, 1995) the assembly of cadherin-containing cell-cell contacts (Takaishi et al., 1997; Braga et al., 1997), cell polarity (Nobes and Hall, 1999; Qiu et al., 2000), and kinase signaling pathways including c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (Coso et al., 1995; Minden et al., 1995).

The mechanism through which Cdc42 exerts its effects on both cytoskeletal rearrangement and kinase signaling pathways is through its interaction with downstream effector proteins. A general feature of Cdc42 effector proteins is the presence of a conserved 16 amino acid Cdc42 binding domain termed a CRIB (Cdc42/Rac Interactive Binding) domain (Burbelo et al., 1995). Cdc42 mediates a diverse array of biological activities because of its interaction with a large repertoire of over 25 effector proteins identified to date. Binding of activated Cdc42 to the CRIB domain of kinase effectors including PAK (Manser et al., 1997; Martin et al., 1995) activates the kinase activity of these proteins (Bagrodia et al., 1995; Brown et al., 1996; Lei et al., 2000). Other non-kinase effectors of Cdc42 such as WASP (Symons et al., 1996) and N-WASP (Miki et al., 1996) link Cdc42 to actin nucleation via the WASP/Arp2/3 complex interaction (Rohatgi et al., 1999; Higgs and Pollard 1999; Prehoda et al., 2000). Taken together, these studies demonstrate that CRIB-containing effector proteins regulate multiple biological activities including cytokeletal organization and kinase signaling.

To identify new signaling molecules that function directly downstream of Cdc42, we searched the NCBI EST database using the CRIB domain of the non-kinase Cdc42 effector proteins MSE55/CEP1 (Burbelo et al., 1999) for cDNAs that shared homology within this region. Using this approach, we identified two genes that comprise a new family of Cdc42 effector proteins. These proteins are designated SPECs for Small Protein Effector of Cdc42. There are two human members of this family, SPEC1 and SPEC2 that are 79 and 84 amino acids respectively. Both SPEC proteins contain a centrally located CRIB domain through which they bind Cdc42. Northern blot data and DNA sequence analysis revealed that there are several splice variants of SPEC1. One of these variants, designated SPEC1-β potentially encodes a 38 amino acid protein, lacking a CRIB

domain, but containing the same first 18 amino acids as SPEC1. Early studies on this work indicated that the ratio of SPEC1 to SPEC1-β isoforms may be altered in more metastatically aggressive breast cancer cell lines, where SPEC1-β is more highly expressed in the highly metastatic MDA-231 and MDA-435 breast cancer cell lines as compared to the less aggressive T47D and MCF-7 breast cancer cell lines. As proposed in our original statement of work, there are three specific aims for this research: 1. We will test the hypothesis that SPEC1 and SPEC1-β proteins affect actin organization, cell shape, and cell movement in breast cancer cells. 2. We will examine the effect of SPEC1 and SPEC1-β on kinase signaling pathways and transcriptional activation. 3. We will test the hypothesis that the levels of SPEC1 and SPEC1-β proteins in different breast cancer cell lines correlate with breast cancer aggressiveness.

Body

Specific Aim 1: We will test the hypothesis that SPEC1 and SPEC1-β proteins affect actin organization, cell shape, and cell movement in breast cancer cells.

Results for Specific Aim1:

We examined the cellular distribution of epitope-tagged SPEC1 expression by immunofluorescence in Cos1 cells. In Cos1 cells, SPEC1 showed diffuse cytoplasmic localization (Figure 1). SPEC-expressing cells did not show altered actin structures or cell morphology in Cos1 cells or in MCF-7 breast cancer cells (data not shown). We next determined whether SPEC1 influenced Cdc42 function when co-expressed with Cdc42. Cos1 cells expressing the constitutively active Cdc42-Q61L mutant exhibited a widely spread and flattened phenotype with some filopodia (Figure 1). Cotransfection of SPEC1 with Cdc42-Q61L resulted in cells that were much less spread than cells expressing Cdc42-Q61L alone (compare Figure 1, C and D with Figure 1A) or untransfected cells (data not shown). In contrast to what was seen with wild type SPEC1, cells coexpressing Cdc42-Q61L and the CRIB domain mutant (SPEC1-H38A) resembled cells transfected with Cdc42-Q61L alone (compare Figure 1, E and F with Figure 1A). These results suggest that SPEC1 modifies Cdc42 function. In these experiments SPEC1 appears to alter Cdc42 activity by binding to it via the CRIB domain. These results suggest that SPECs may function to block the interaction of Cdc42 with other effector proteins, although we can not rule out the possibility that the observed blocking activity was due to overexpression of SPEC1 protein.

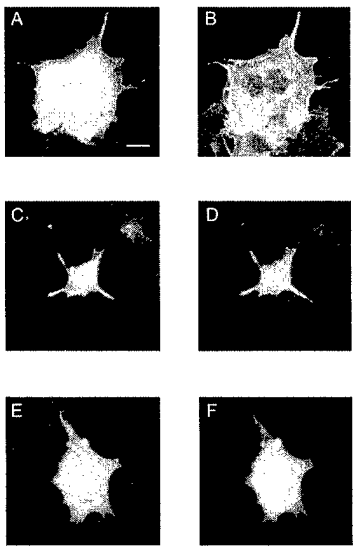

Figure 1: SPEC1 alters Cdc42 activity in Cos1 cells

Since SPEC1 expression did not noticeably alter the morphology of Cos1 cells or MCF-7 breast cancer cells, we studied the effects of SPEC1 expression in NIH-3T3 fibroblasts. In NIH-3T3 fibroblasts, SPEC1 displayed a predominant cortical localization (Figure 2) and frequently, these transfected cells showed extensive membrane blebbing (Figure 2). F-actin stained strongly within the periphery of the blebs, but not within the blebs (Figure 2). Expression of SPEC2 also showed the same cortical localization, membrane blebbing and F-actin staining phenotype (Figure 2, C and D). A similar pattern of cortical staining and blebbing were observed with a myc epitope tag located either at the N- or C-terminus of SPEC1 and using a 20-fold range of plasmid concentrations (100 ng to 2 μg; data not shown). Although this SPEC-induced membrane blebbing is morphologically similar to the membrane blebbing associated with apoptosis, there is no functional association of the SPEC-induced

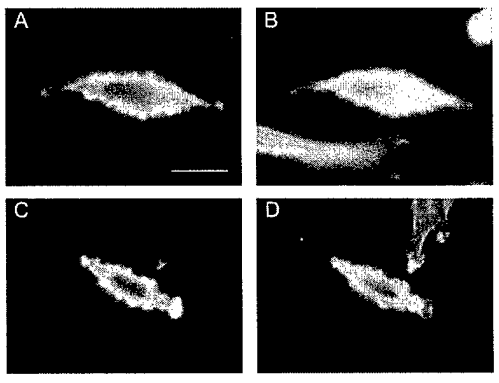

Figure 2: SPEC1 and SPEC2 induce membrane blebbing in NIH-3T3 ifibroblasts

blebbing with apoptosis. That is, neither nuclear condensation following DAPI staining of nuclei nor annexin–V positive staining, a marker for phosphatidylserine flipping in the membrane, was observed in these transfected cells (data not shown).

Quantitatively, membrane blebbing was observed in 40-60% of the Flag epitope-tagged SPEC1 transfected cells but only in about 5% of cells expressing the vector-alone control (Figure 3). We also used a bicistronic expression vector, expressing both SPEC1 and Enhanced Green Fluorescent Protein (EGFP) from the same vector, to rule out the possibility that the epitope tags might influence SPEC function. About 40% of the cells expressing the bicistronic SPEC1 construct showed a blebbing phenotype, while only 10% were blebbing with the EGFP-alone vector (Figure 3). Taken together, these results demonstrate that expression of SPECs, whether epitope-tagged or untagged leads to membrane blebbing in NIH-3T3 fibroblasts.

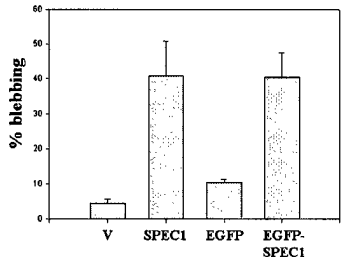

Figure 3: Quantitative analysis of the effect of tagged and untagged SPEC1 on blebbing

In order to determine whether any of the three conserved regions in SPECs are necessary for SPEC1-induced membrane blebbing, we examined the phenotype of cells transfected with various SPEC1 mutants. An additional, N-terminal mutant, SPEC1-C10A, C11A was created within two conserved cysteine residues because of the potential role of these residues in lipid modification or protein interactions. Using cell counting it was found that both the positive and negative controls gave the expected results: approximately 44% of N-terminal Flag-tagged SPEC1-transfected cells blebbed, as compared to only 4% using a vector-alone control (Figure 4). The C-terminal double point mutant, SPEC1-Q62A,K66A, had no effect on the level of blebbing (Figure 4). In contrast, the cells expressing the N-terminal mutant (SPEC1-C10A, C11A), which showed a similar level of expression and cortical localization, produced the blebbing phenotype in only 20% of the transfected cells (Figure 4). Single or triple amino acid substitutions within the CRIB domain of SPEC1 resulted in somewhat fewer blebs, although they still produced significantly more than the negative controls (30% vs. 4%; Figure 4). Additional studies expressing a dominant negative mutant of Cdc42 (Cdc42-T17N) alone did not induce membrane blebbing and co-expression with SPEC1 did not block membrane blebbing (data not shown). These results support a model whereby SPEC1-induced blebbing does not occur through classical Cdc42-effector interactions and suggest that SPEC1 may act independently of Cdc42 or perhaps upstream of Cdc42 to induce membrane blebbing. These data also confirm that SPEC-induced membrane changes are not directly due to sequestration of Cdc42.

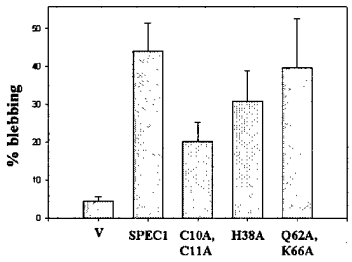

Figure 4: Activity of different SPEC1 mutants on inducing membrane blebbing

To define the relationship between Cdc42 activity and SPECs, we tested the effect of SPEC1/Cdc42 co-expression in NIH-3T3 fibroblasts. In fibroblasts, expression of Cdc42L61 resulted in cells that predominantly exhibited a membrane ruffling phenotype, possibly through activation of Rac signaling. We cotransfected SPEC1 or the SPEC1-CRIB mutants (SPEC1-H38A or SPEC1-P33A,H38A, H41A) with constitutively active Cdc42 and quantified the number of transfected cells showing a ruffling phenotype. Expression of a constitutively active Cdc42 mutant (Cdc42-Q61L), but not wild type Cdc42 (data not shown), in NIH-3T3 fibroblasts induced membrane ruffling in 52% of the transfected cells (Figure 5, A and B). Co-transfection of SPEC1 blocked

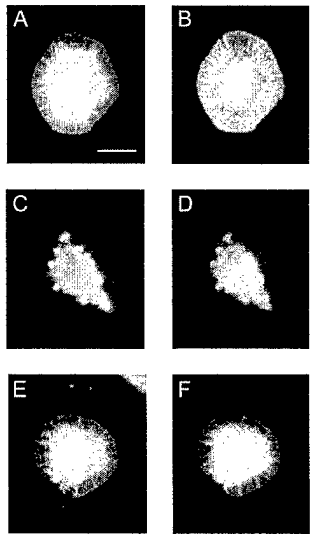

Figure 5: SPEC1 alters Cdc42-induced cell shape changes

ruffling in all but 5% of the transfected cells and increased the number of blebbing cells (Figure 5, C and D). Coexpression of the SPEC-H38A mutant resulted in 34% of the cells showing a membrane ruffling phenotype (Figure 5, E and F) and resembled cells transfected with Cdc42 alone (compare Figure 5, A and B with Figure 5, E and F). Similar results were obtained with the SPEC1-P33A,H38A,H41A CRIB mutant (data not shown). In these cotransfections experiments, SPEC1 and Cdc42 proteins appear to localize to similar regions within the cells, suggesting that SPECs and Cdc42 may be contained within the same signaling complexes (Figure 5). As with Cos1 cells, these transfections demonstrate that SPEC1 expression led to an altered Cdc42-induced morphology and that this alteration is dependent on the presence of an intact CRIB domain.

Although we were able to obtain high levels of SPEC1 expression in several different mammalian cell types, we were not able to observe consistenly high expression of SPEC1-β. In the attempted SPEC1-β transfections, only very few cells were expressing the SPEC1-β protein, however, in these cells the overall morphology of the cells and actin cytoskeletal organization was apparently normal (data not shown). Despite this particular setback, these studies overall show that SPEC1 overexpression alters or reverses the cellular morphologies produced when Cdc42 is overexpressed in Cos1 cells and in NIH-3T3 fibroblasts. The membrane blebbing induced by SPEC1 overexpression in NIH-3T3 fibroblasts was not observed in Cos1 cells, possibly due to quantitative differences in expression levels of SPEC proteins between the two cell types. Nevertheless, these results show that SPECs are capable of modifying Cdc42-dependent signaling at both the biochemical and cellular levels in a CRIB-dependent manner. SPEC binding may prevent the interaction of Cdc42 with other effector proteins. Consistent with this model, a polypeptide containing just the CRIB domain of PAK can effectively inhibit Cdc42 activation of JNK kinase (Minden et al., 1995) and block transcriptional activation (Osada et al., 1997), while a polypeptide containing the CRIB domain of ACK-1 can act as a Cdc42-specific inhibitor, blocking v-Ha-Ras-induced transformation (Nur-E-Kamal et al., 1999).

However, we do not know if the specific biochemical and biological effects observed here, with overexpressed SPECs, reflect the normal function of these small proteins. In particular, SPEC overexpression induced membrane blebbing in NIH-3T3 fibroblast, which was not blocked by dominant negative Cdc42 expression. Despite these findings, it is still possible that SPECs function in Cdc42-induced morphological changes, since a dominant negative approach may not rescue the abnormal morphology of overexpressed SPEC protein. Furthermore, various studies have shown that non-apoptotic membrane blebs function normally in cell spreading (Erickson et al., 1976; Cunningham, 1995) and locomotion (Trinkaus, 1980; Keller and Eggli, 1998; Cunningham et al., 1992). Mechanistically membrane blebs occur at sites where the cortical actin is locally depolymerized or detached from the membrane (Cunningham, 1995; Keller and Eggli, 1998; Cunningham et al., 1995) via alteration in cortical actin binding proteins (Keller and Eggli, 1998), myosin light chain kinase activity (Mills et al., 1998; Huot et al., 1998) and/or focal complex assembly (Huot et al., 1998). Thus, it is possible that SPEC1 and SPEC2 may function as classical Cdc42 effector proteins by altering the normal signaling pathways leading to actin, myosin and/or focal complex assembly.

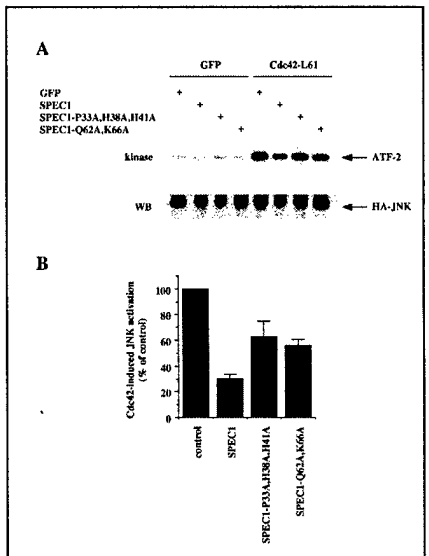

Figure 6: SPEC1 inhibits Cdc42-induced JNK activation

Specific Aim 2: We will examine the effect of SPEC1 and SPEC1-β on kinase signaling pathways and transcriptional activation.

Results for Specific Aim 2:

Since a variety of studies have shown both that Cdc42 (Coso et al., 1995; Minden et al., 1995) and some Cdc42 effector proteins (Teramoto et al., 1996; Bagrodia et al., 1995; Zhang et al., 1995; Brown et al., 1996) can activate JNK activity, we tested SPEC1 and several SPEC1 mutants for their effect on Cdc42-induced JNK activation. First, an expression construct of human SPEC1 carrying an N-terminal FLAG-epitope tag was transfected

into NIH-3T3 fibroblasts and its expression analyzed by Western blotting using a monoclonal antibody against the N-terminal FLAG epitope tag. Using this approach, SPEC1 was detected in lysates as an ~8 kD species (data not shown), of which about 1 kb is contributed by the epitope tag. Second, several SPEC1 mutants were constructed including two CRIB mutants and a C-terminal double mutant. The two CRIB mutants, SPEC1-H38A and SPEC1-P33A,H38A,H41A, contain alanine substitutions within critical contact sites known to be involved in Cdc42 binding (Abdul-Manan et al., 1999; Mott et al., 1999). The third mutant, SPEC1-Q62A,K66A, contained mutations within the 9 amino acid region conserved between both SPEC proteins that might be part of an extended high affinity Cdc42 binding site. Cos1 cells were cotransfected with the expression vectors for GFP (control) or Cdc42-Q61L, hemagglutinin (HA) epitope-tagged JNK and Flag-tagged SPEC1 constructs. After 24 hours, transiently expressed HA-JNK was isolated by immunoprecipitation and used for *in vitro* kinase assays. All SPEC1 constructs tested were not able stimulate JNK activity on their own (Figure 6A). Expression of Cdc42 with HA-JNK stimulated kinase activity (Figure 6, A and B). Cotransfection of cells with wild-type SPEC1 significantly reduced the Cdc42-induced JNK activation (Figure 6, A and B). In contrast, SPEC1-P33A,H38A,H41A or SPEC1-Q62A,K66A were markedly less effective at blocking Cdc42-induced JNK activation (Figure 6, A and B). In addition, a similar failure to block Cdc42-induced JNK activation was also observed with the single CRIB domain mutant (data not shown). Although we cannot rule out the possibility that our overexpression studies have resulted in abnormally high levels of SPEC1, which may non-specifically inhibit Cdc42 signaling pathways, these results may also suggest that SPEC1 modulates JNK activity by binding or sequestering Cdc42 through a CRIB-dependent interaction.

Specific Aim 3: We will test the hypothesis that the levels of SPEC1 and SPEC1-β proteins in different breast cancer cell lines correlate with breast cancer aggressiveness.

Results for Specific Aim 3:

Our preliminary data suggested that only highly metastatic breast cancer cell lines expressed the SPEC1-β mRNA. Thus, SPEC1-β protein expression may be a marker of highly metastatic breast cancer cells. In order to test this possibility we first performed Northern blot analysis of SPEC1 to examine its expression in a variety of tissues. Using Northern blot analysis with a probe derived from the 3'-untranslated region of the 1.2 kb SPEC1 cDNA (Pirone et al., 2000), we detected the ubiquitous expression of three major transcripts of 1.6, 3.3, and 6.3 kb in brain, uterus, placenta, kidney, spleen and lung (Figure 7A). In these tissues a minor transcript of 10.2 kb was also detected (Figure 7A). To rule out non-specific hybridization, a second probe directed against the 5'-untranslated region of SPEC1 was used in a matched blot. Hybridization with this alternate probe showed an identical distribution pattern to that seen with the 3'-untranslated probe (Figure 7B). Northern analysis using a probe derived from the coding region of SPEC1 also detected the same pattern of mRNA transcripts (data not shown). This Northern data suggests that the different sized SPEC1 mRNA transcripts are derived from alternative splicing. Furthermore, the Northern results seen with the three independent probes demonstrate that the 1.6 kb, 3.3 and 6.3 kb mRNA transcripts all share the originally determined 1.2 kb SPEC1 cDNA sequence.

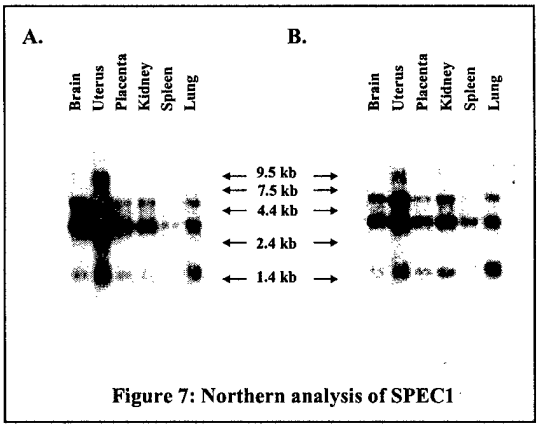

Figure 7: Northern analysis of SPEC1

In an effort to identify the larger SPEC1 mRNA species, we searched the EST database for cDNA clones that overlapped the previously determined 1.2 kb cDNA sequence of SPEC1. Identification and sequencing of several new overlapping SPEC1 cDNA clones revealed an additional 1.8 kb of 3'-untranslated sequence that contained no additional open reading frames. Sequence analysis of several different SPEC1 cDNA clones revealed that they utilized one of two different polyadenylation signals; one located at nucleotides 1282-1286 and the other at nucleotides 2987-2991 (Figure 8). These results support the data from the SPEC1 Northern

analysis and confirm that the 1.6 kb and 3.3 kb SPEC1 mRNA species are due to alternate usage of these polyadenylation sites.

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   1  CAGAGCTAGCCCGGGAAGCCCACACTGGCGGCCACGGAGCAGAGTCCCTCACCCCCACCAGCTGTAGCTGAACGTCTGGATGGTGGAGAA
  91  GAGCAGGGTTCCGAGTCTGAGGAAGACATAACCTTGTGCCTGCCTGCCCACCTCTCTCTCTGGTCCTGTTCATCTCTCAGGCTCTGAGAC
 181  ACTGACCTTCACTGCTCAGTTAAAGGTTCCAGGGATTCCACTTTGTCTGGACCCATCCAGCTGAGTGAACCCAGGGTGGTGGTGTATCTG
 271  GGGAGAGTGAGGAGTGGGTTGTCCAAACACCAGGGAAAGAGCCCTTTGGGGCCTCAGACAGAGGAGTGAAGCTGGAACCATCAGGGAACA
                                                                                          M   1
 361  TGAGTGAATTTTGGCACAAACTGGGCTGCTGTGTGGTAGAGAAACCCCAGCCGAAGAAGAAGAGAAGACGGATTGACCGGACCATGATTG
        S   E   F   W   H   K   L   G   C   C   V   V   E   K   P   Q   P   K   K   K   R   R   R   I   D   R   T   M   I   G  31
 451  GGGAACCAATGAATTTTGTTCACCTGACTCACATTGGCTCAGGGGAGATGGGGGCCGGAGATGGACTTGCCATGACAGGTGCAGTTCAGG
        E   P   M   N   F   V   H   L   T   H   I   G   S   G   E   M   G   A   G   D   G   L   A   M   T   G   A   V   Q   E  61
 541  AGCAGATGAGATCCAAGGGAAACCGAGATAGGCCATGGAGCAATTCTAGGGGCTTATAGCTCCAATAATGGAATGGTTCTGCCATCTTGA
        Q   M   R   S   K   G   N   R   D   R   P   W   S   N   S   R   G   L   *                  79
 631  AACCCCCATTCTGTTTCCAGCCCAGAAGAAATGCTGCCCCTACCAGATCCCTCCTTGAACCAGTGATCTAAGGACCCCTCTTTTCCCTAT
 721  CTGCCTAACAGTGCCTCACAAGGCTTGGGGGCTGGACTCCCTCTACTCCCTCTGGCCATAGCCCCTCCTGGAGATGGGGTCAAGGCAGCA
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2791  CCTAGAAATCTCTGGTCACCGTGATCTTCGGCCTCATTCTAATACCTGTTCTTTGGACAGTCTTTTTCCTTTGGTGCTCTCTTGCCTTTA
2881  GCTACCTTCTCTAATATGTATGCTACCATCACTAATAAAGTGATGGGAATGGGTTTGAGAGTCGTAATTTATATTAAAAAGTTGTTGGAC
2971  TTTTAAATACATTTTTTCAAATAAAAAATTAAGCAAAAT
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Figure 8: Nucleotide and amino acid sequences of the human SPEC1 cDNA

In addition to the 3'-end alternatively spliced SPEC1 clones, we also verified the sequence of the SPEC1-β splice variant. If translated, the SPEC1-β translation product would encode a 38 amino acid protein, in which the first 18 amino acids would be identical to SPEC1 (Figure 9A). SPEC1-β would also contain a unique proline rich C-terminus, ending in a CAAX box-like sequence (Figure 9A). Specifically the C-terminal, C-A-V-R sequence of SPEC1-β may be involved in plasma membrane targeting as in other proteins such as Ras (Hancock et al., 1991; Moores et al., 1991).

To examine the distribution of the SPEC1-β mRNA transcript, we performed RT-PCR with human cDNAs derived from lung, placenta, heart, kidney, liver, and colon. In these experiments, we used two different sets of primers to amplify either the SPEC1 or SPEC1-β cDNAs. Using RT-PCR, we obtained the expected 230 bp fragment corresponding to SPEC1 in all tissues examined (Figure 9B). In the case of SPEC1-β detection, we designed primers spanning intron 3 to differentiate between the SPEC1-β cDNA and potential genomic contamination. Using these SPEC1-β-specific primers, a 720 bp fragment corresponding to the SPEC1-β cDNA was amplified in all of the same tissues (Figure 9B). In each case, SPEC1 and SPEC1-β products were confirmed using Southern blotting (data not

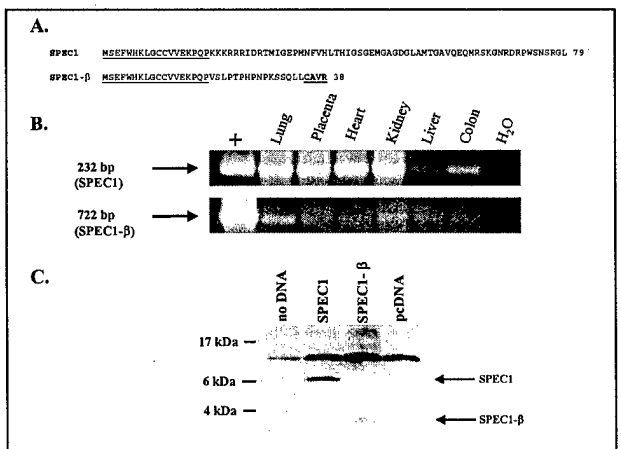

Figure 9: Protein sequence and expression of SPEC1-β

shown). Based on experiments using primers common to both SPEC1 and SPEC1-β, we consistently observed a much lower level of SPEC1-β expression as compared to SPEC1 (data not shown). These results confirm the presence of SPEC1-β, but suggest that it is present only as a minor transcript.

Because of the unusually small size of the SPEC1-β mRNA transcript, we used *in vitro* transcription/translation to examine whether the SPEC1-β cDNA could generate a protein product. In these experiments, plasmid constructs were generated containing SPEC1 or SPEC1-β cDNAs downstream of the T7 promoter. In this *in vitro* transcription/translation system, the incorporation of biotinylated lysine residues was used to monitor protein production. Analysis of the translation products on an 18% SDS-PAGE gel revealed that SPEC1 and SPEC1-β generated 8 and 4 kDa protein species, respectively (Figure 9C). In contrast, no protein product was observed with the sample containing no DNA or with the empty vector control (Figure 9C). These results support the possibility that the SPEC-β protein is translated *in vivo*.

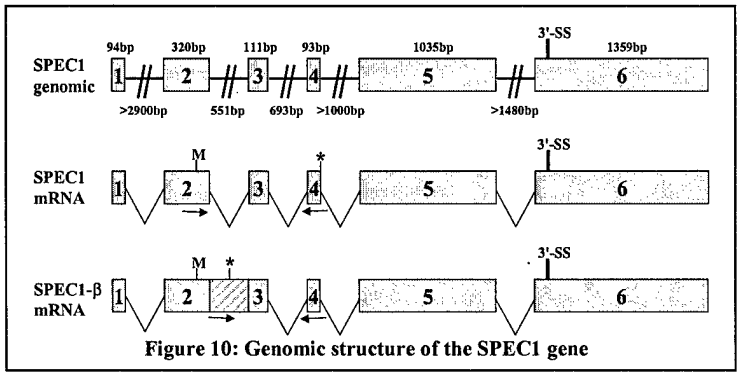

Figure 10: Genomic structure of the SPEC1 gene

To further understand the organization of the SPEC1 gene and the origin of the mRNA splice variants, a single BAC clone was isolated from a genomic library with a SPEC1-specific probe. While this work was in progress, the working draft sequences of a bacterial artificial chromosome clone (RP11-316M1) containing the SPEC1 gene became available through the Human Genome Project and we have used these data in conjunction with our sequence data to determine the exon-intron organization of the SPEC1 gene. Characterization of the SPEC1 BAC clone by DNA sequencing and PCR analysis revealed that both the entire 1.6 kb and 3.3 kb SPEC1 cDNAs were encoded by six exons (Figure 10). Of these 6 exons, only exons 2, 3 and 4 encoded the 79 amino acid residues of the SPEC1 protein. Exon 2, consisting of 320 base pairs, contained the start methionine and the first 18 amino acids of the SPEC1 coding sequence (Figure 10). Exon 3 was 111 base pairs and encoded the entire CRIB domain responsible for Cdc42 binding, while exon 4 encoded the C-terminal 24 amino acids of SPEC1 and was 93 base pairs long (Figure 10). The difference in the 3'-ends of the 1.6 kb and 3.3 kb species was due to alternative splicing in exon 6, whereby an additional 1.8 kb of 3'-untranslated sequence generates the 3.3 kb cDNA. We also compared the SPEC1-β cDNA sequence with the genomic sequence of SPEC1 to formally prove that the SPEC1-β cDNA was derived from the SPEC1 gene. Comparison of these sequences revealed that the 0.5 kb intron located between coding exons 2 and 3 was retained in the SPEC1-β cDNA, although other introns in the SPEC1 gene were properly spliced (Figure 10). Finally, using fluorescent in situ hybridization the SPEC1 gene was localized to human chromosome 1q21.1-1q21.3, consistent with the sequence and mapping data of the human genome project (data not shown).

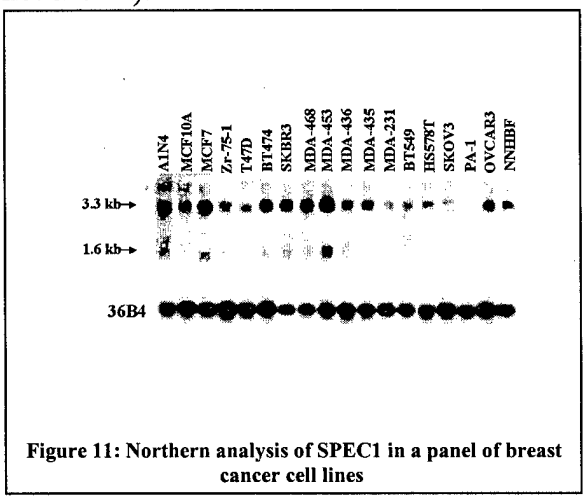

Figure 11: Northern analysis of SPEC1 in a panel of breast cancer cell lines

To address whether there were differences in the various SPEC1 mRNA transcripts in breast cancer cells, we performed a northern blot using RNA from a panel of breast cancer cell lines, where the cell lines represented were placed in order of increasing metastatic aggressiveness and decreasing ER status (Figure 11). Also included in this panel were three ovarian cancer cell lines on the far right. In this blot, the predominant SPEC1 mRNA species appears to be the 3.3 kb mRNA species and interestingly, while the smaller 1.2 kb mRNA species appears to have a more variable expression pattern. Interestingly, the SPEC1 message may decrease with increasing

metastatic aggressiveness, which may suggest that loss SPEC1 mRNA expression correlates with increased metastatic aggressiveness (Figure 11).

Additional work completed outside the scope of original Specific Aims:
Generation and characterization of SPEC1 polyclonal antibodies:

In order to generate polyclonal antibodies to SPEC1, we utilized a peptide corresponding to the C-terminal 17 amino acids of SPEC1: MRSKGNRDRPWSNSRGL. This peptide was produced as a cysteine conjugated peptide (Research Genetics) for ease in making subsequent KLH conjugates and peptide affinity columns. After conjugation with KLH, the peptide-KLH conjugate was sent to Rockland, Inc. for immunization into two different rabbits. The test bleeds resulting from these immunizations were affinity purified using a SPEC1 peptide affinity column.

In order to determine whether the affinity purified serum contained antibodies that were immunoreactive to SPEC1, MCF-7 cells were lysed in SDS-PAGE sample buffer and run on a 4-20% Tris-glysine gel. After blotting to nitrocellulose, the membrane was probed with the SPEC1 rabbit polyclonal antibody, followed by an anti-rabbit-HRP conjugated secondary antibody. In this experiment, MCF-7 whole cell lysates showed an 8 kDa molecular weight species that strongly interacted with the SPEC1 rabbit polyclonal antibody (Figure 12). Also shown is recombinant GST-SPEC1, which was also detectable with the affinity purified SPEC1 antibody (Figure 6.1). To rule out the possibility that the 8 kDa molecular weight species was non-specific cross reactivity with the secondary antibody, we probed a duplicate blot with anti-rabbit-HRP conjugated secondary antibody alone, but observed no cross-reactivity at the 8 kDa molecular weight (data not shown). These results show that the SPEC1 rabbit polyclonal antibody can detect both endogenous SPEC1 protein from mammalian cell lysates as well as a recombinant GST-SPEC1 fusion protein produced in bacteria.

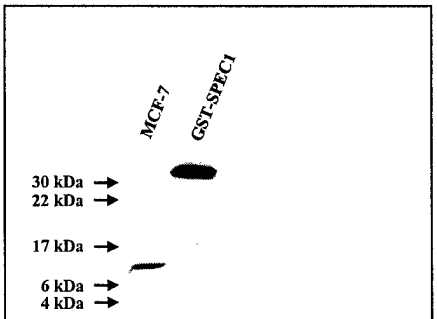

Figure 12: The SPEC1 rabbit polyclonal antibody detects both endogenous and recombinant GST-SPEC1 protein.

Similar Western blotting experiments using HeLa whole cell lysates revealed that endogenous SPEC1 was not detectable in these lysates (data not shown). Based on the hypothesis that HeLa cells contain only very low levels of the SPEC1 protein, we wanted to determine whether transfection of a mammalian expression vector containing the SPEC1 cDNA (pcDNA3-SPEC1) could yield an increase in SPEC1 protein expression. HeLa cells were transfected with a mammalian expression vector containing the SPEC1 cDNA. Twenty-four hours after transfection, the HeLa cells were lysed in SDS-PAGE sample buffer and western blotting was performed using the SPEC1 rabbit polyclonal antibody. As a control, untransfected HeLa cell lysates were also analyzed on the same blot. Here, we were able to detect the SPEC1 protein only in the HeLa cells that were transfected with the SPEC1 cDNA (Figure 13). These results provide further support that the SPEC1 rabbit polyclonal antibody interacts specifically with the SPEC1 protein and that SPEC1 protein expression can be increased by transfection with SPEC1 DNA.

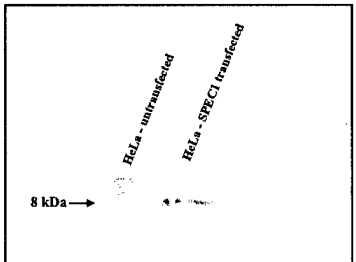

Figure 13: SPEC1 is detectable in transfected HeLa lysates.

The SPEC1 polyclonal antibody was also tested for its ability to detect endogenous SPEC1 protein by immunofluorescence. In these experiments both MCF-7 and HeLa cells were plated onto glass coverslips. After attachment, the cells were fixed, permeabilized, and stained using the affinity purified SPEC1 polyclonal antibody. A vesicular staining pattern was observed in both MCF-7 and HeLa cells (data not shown). This pattern of staining was able to be competed with a pre-incubation with the SPEC1 peptide indicating that the vesicular staining was specific for a SPEC1-like epitope (data not shown). We cannot, however, rule out the possibility that this vesicular staining pattern is due to cross-reactivity with a similar epitope on another protein.

The observed vesicular staining pattern was not altered by co-expression of Cdc42, or by treatment with bradykinin, which suggests that the vesicles may not be SPEC1 (data not shown). If the vesicles did represent endogenous SPEC1 staining, then activation of Cdc42 might be expected to cause SPEC1 redistribution from the vesicular compartment, which it does not.

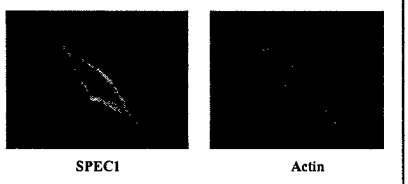

Figure 14: Overexpressed SPEC1 protein shows both cytoplasmic and membrane localization.

In order to determine if overexpression of SPEC1 was detectable by immunofluorescence using the SPEC1 polyclonal antibody, we transfected HeLa cells with a mammalian expression vector containing the SPEC1 cDNA. Twenty-four hours post-transfection the cells were fixed and processed for immunofluorescence using the SPEC1 antibody. HeLa cells overexpressing SPEC1 showed a cytoplasmic and membrane staining pattern (Figure 14). This membrane localization of SPEC1 might be expected based on its ability to interact with Cdc42. Together, these data indicate that the SPEC1 polyclonal antibody does not detect endogenous SPEC1 protein by immunofluorescence, but does detect overexpressed SPEC1 protein.

Examination of phospholipid binding capability of SPEC1:

Although SPEC1 contains a centrally located CRIB domain involved in Cdc42 binding, the functional significance of other regions of the SPEC1 molecule remain undefined. Interestingly, a basic amino acid sequence preceding the CRIB domain of SPEC1 resembles a basic stretch of amino acid residues preceding the CRIB domain in N-WASP that is involved in PIP2 binding (Higgs and Pollard, 1999; Prehoda et al., 2000). This region in SPEC1 consists of nine amino acid residues, K-K-K-R-R-R-I-D-R, that is similar to basic amino acid sequences sequences found in other known phospholipid binding proteins including gelsolin, villin and ezrin.

A fat western filter-binding assay was used to determine whether SPEC1 could bind phospholipids (Stevenson et al., 1998; Dowler et al., 1999). In this assay, nitrocellulose membranes spotted with 100 pmol of 12 different phospholipids (Echelon Research Lab) were incubated with GST-SPEC1, GST-SPEC1-del1 (amino acids 2-27), or GST recombinant proteins. After membrane washing the bound recombinant proteins were detected using GST antibodies followed by ECL development. GST-SPEC1 strongly bound phosphatidylinositol (PtdIns)-3-P, PtdIns-4-P, PtdIns-5-P, phosphatidic acid, and PtdIns-(3,5)P_2, weakly bound PtdIns-(3,4)P_2, PtdIns-(4,5)P_2 and PtdIns-(3,4,5)P_3 but did not bind phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphaditylinositol (Figure 15). A similar pattern of reactivity to that seen with GST-SPEC1 was also observed with 6X-His-tagged SPEC1 (data not shown), indicating that neither the GST nor the 6X His tag interfered with SPEC1-phospholipid binding. To determine whether the basic region of SPEC1 was responsible for PIP binding we utilized a deletion mutant of SPEC1 containing the N-terminus of the protein (amino acids 1-29). This short peptide containing the basic region of SPEC1 showed the same profile as the full length SPEC1 protein confirming that the basic region was likely required for binding the different phospholipids (Figure 15). GST alone was found to only weakly interact with a subset of the phospholipids but this is likely to be non-specific binding due to the high concentrations of protein that were used in the experiments. Together this experimental data supports the possibility that SPEC1 binds different phospholipids than and these phospholipids, like with N-WASP, may control either the activity or the localization of the SPEC1 protein.

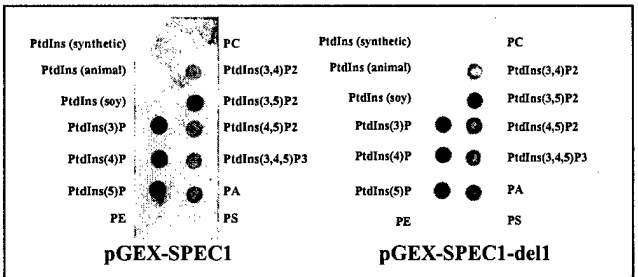

Figure 15: Interaction of SPEC1 with phospholipids.

An analysis of the relative strengths of these interactions showed that SPEC1 interacts most strongly with the PtdIns-monophosphates (Figure 16). Thus, in these experiments we have determined that SPEC1 strongly interacts with a number of phospholipids, including PtdIns-(3)P, PtdIns-(4)P, PtdIns-(5)P, phosphatidic acid and PtdIns-(3,5)P_2. Furthermore, SPEC1 interacted more weakly with PtdIns-(3,4)P_2, PtdIns-(4,5)P_2, and PtdIns-(3,4,5)P_3. An analysis of the relative strengths of these interactions showed that SPEC1 interacts most strongly with the PtdIns-monophosphates. Previously, the non-kinase Cdc42 effector N-WASP was shown to interact with PtdIns-(4,5)-P_2 (Rohatgi et al., 1999; Prehoda et al., 2000). Based on the ability of SPEC1 to bind to a variety of different phospholipids, we also tested whether N-WASP would bind to the same phospholipids as SPEC1. These studies indicate that N-WASP does, in fact, share the same phospholipid binding profile as SPEC1, indicating that N-WASP potentially binds many more phospholipids than previously known (Pirone, Kisailus, Burbelo unpublished observations). These data provide support for the idea that SPEC1 and N-WASP proteins may be regulated by similar mechanisms.

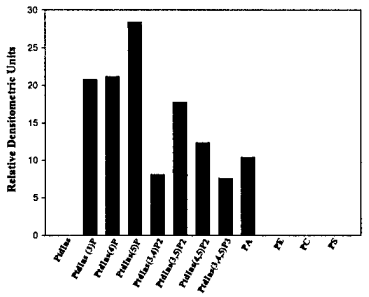

Figure 16: SPEC1 interacts strongly with the PTDIns-monophosphates.

Key Research Accomplishments

- We have tested the hypothesis that SPEC1 and SPEC1-β proteins affect actin organization and cell shape. In NIH-3T3 fibroblasts we have found that SPEC1 induces the formation of plasma membrane blebbing that was not associated with apoptosis. Furthermore, we have shown that SPEC1 expression alters Cdc42 activity in both Cos1 cells and in NIH-3T3 fibroblasts and that an intact CRIB domain is required for this effect.

- We have examined the effect of SPEC1 on the JNK signaling pathway. In these experiments we have determined that SPEC1 expression does not stimulate JNK activity on its own and when co-expressed with Cdc42, SPEC1 significantly reduced Cdc42-induced JNK activation. This ability of SPEC1 to downregulate JNK activity required both an intact CRIB domain and C-terminus, as mutations in these regions were less effective and blocking Cdc42-induced JNK activation.

- Using Northern blot analysis, we have determined that the SPEC1 mRNA is ubiquitously expressed and is present in multiple molecular weight species of 1.3, 3.3, 6.3, and 10.2 kb, suggesting complex alternative splicing.

- We have determined that the 1.2 kb and 3.3kb molecular weight transcripts of SPEC1 differ in their 3′-untranslated regions and arise via alternate polyadenylation.

- In addition to the 3′-end alternatively spliced SPEC1 clones, we also verified the sequence of the SPEC1-β splice variant and examined its distribution by RT-PCR in several human tissues including lung, placenta, heart, kidney, liver, and colon. In these experiments, we detected both SPEC1 and SPEC1-β transcripts in each tissue examined, however, we consistently observed a much lower level of SPEC1-β expression as compared to SPEC1, suggesting that SPEC1-β may be present as only a minor transcipt.

- We have determined the genomic sequence of the SPEC1 gene and have found that it is composed of six exons of which exons 2, 3, and 4 contribute to the coding sequence of the SPEC1 protein.

- Comparison of the SPEC1-β cDNA sequence with the SPEC1 genomic sequence revealed that SPEC1-β was generated by an intron read-through event.

- To address whether there were differences in the various SPEC1 mRNA transcripts in breast cancer cells, we used Northern blotting with RNA from a panel of breast cancer cell lines. In this experiment, we found that the predominant SPEC1 mRNA species appears to be 3.3 kb and that the SPEC1 message may decrease with increasing metastatic aggressiveness.

- We have generated and characterized a polyclonal antibody to SPEC1. These antibodies are suitable for Western blotting. They are also able to detect SPEC1 by immunofluorescence in SPEC1 overexpressing cells, however, they are not able to detect endogenous SPEC1 by immunofluorescence.

- Based on known phospholipid binding capabilities of other non-kinase Cdc42 effector proteins such as the WASP family members, we explored the possibility that SPECs might also interact with phospholipids. Using *in vitro* binding assays with recombinant SPEC1 protein, we have shown that SPEC1 strongly bound phosphatidylinositol (PtdIns)-3-P, PtdIns-4-P, PtdIns-5-P, phosphatidic acid, and PtdIns-(3,5)P_2, weakly bound PtdIns –(3,4)P_2, PtdIns-(4,5)P_2 and PtdIns-(3,4,5)P_3, and did not bind phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphaditylinositol.

Reportable Outcomes

Publications

1. **Pirone, D.M.**, Fukuhara, S., Gutkind, J.S., Burbelo, P.D. (2000) SPECS, small binding proteins for Cdc42. *J. Biol. Chem.* 275: 22650-22656.
2. **Pirone, D.M.**, Carter, D.E., Burbelo, P.D. (2001) Evolutionary expansion of CRIB-containing Cdc42 effector proteins. *Trends in Genetics* 17: 370-373.
3. **Pirone, D.M.**, Oberst, M.D., Carter, D.E., and Burbelo, P.D. (2001) The genomic structure of the human SPEC1 gene reveals complex splicing and close promoter proximity to the AF1q translocation gene. *Gene* 273(2): 295-303.

Abstracts

1. **Pirone, D.**, Fukuhara, S., Gutkind, S., Burbelo, P. Small Binding Proteins Regulate Cdc42 Signaling. (abstract for 91st American Association for Cancer Research annual meeting)
2. **Pirone, D.**, Oberst, M., and Burbelo, P. Potential role of SPEC1 as a Cdc42 adapter molecule involved in integrin assembly and signaling. (abstract for 92nd American Association for Cancer Research annual meeting)

Honors or Awards

2001	Second Place Award in Georgetown University Student Research Days Poster Competition
2001	Finalist in Lombardi Cancer Center Research Fair
2000	First Place Award in Lombardi Cancer Center Research Fair

Invited seminars on research topic

February 2001	Mount St. Marys College, Emmitsburg, MD. "Control of the cytoskeleton by Cdc42 signaling pathways"
March 2001	Georgetown University, Student Research Days Oral Competition. "Potential role of SPEC1 as a Cdc42 adapter molecule involved in integrin assembly and signaling"
March 2001	Laboratory of Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD. "Role of SPECs in Cdc42 signaling pathways"
May 2001	Yale University School of Medicine, Department of Internal Medicine Section of Medical Oncology, New Haven, CT. "Functional analysis of the SPEC family of proteins and their role in Cdc42 signaling pathways."
August 2001	Johns Hopkins University School of Medicine, Department of Biomedical Engineering, Baltimore, MD. "SPEC1: a link between Cdc42 and integrins?"

Degrees obtained when supported by this award

Ph.D. in Tumor Biology from Georgetown University

Employment received based on experience/training supported by this award

Post-doctoral fellowship position received from Johns Hopkins University in the Biomedical Engineering Department

Conclusions

To date, we have made significant progress on the specific aims set forth in the research proposal. First, we have addressed whether SPEC1 and SPEC1-β proteins affect actin organization and cell shape. From these studies, we have determined that in NIH-3T3 fibroblasts, SPEC1 induces the formation of plasma membrane blebbing that was not associated with apoptosis. Mechanistically, membrane blebs occur at sites where the corical actin is locally depolymerized or detached from the membrane (Cunningham, 1995; Keller and Eggli, 1998, Cunningham et al., 1992) via alteration in cortical actin-binding proteins (Keller and Eggli, 1998), myosin light chain kinase activity (Mills et al., 1998; Huot et al., 1998) and/or focal complex assembly (Huot et al., 1998). It is likely that SPECs function as classical Cdc42 effector proteins by altering the normal signaling pathways leading to actin, myosin, and/or focal complex assembly. Furthermore, we have shown that SPEC1 expression alters Cdc42 activity in both Cos1 cells and in NIH-3T3 fibroblasts and that an intact CRIB domain is required for this effect. SPEC1 also affects the JNK signaling pathway, where SPEC overexpression significantly reduced Cdc42-induced JNK activation. This ability of SPEC1 to downregulate JNK activity required both an intact CRIB domain and C-terminus, as mutations in these regions were less effective and blocking Cdc42-induced JNK activation. Together, these results show that SPECs are capable of modifying Cdc42-dependent signaling at both the biochemical and cellular levels.

We have also examined the mRNA distribution pattern of SPEC1. Using Northern blot analysis, we have determined that the SPEC1 mRNA is ubiquitously expressed and is present in multiple molecular weight species of 1.3, 3.3, 6.3, and 10.2 kb, suggesting complex alternative splicing. For at least two of the molecular weight species, the SPEC1 mRNA transcripts differ in their 3′-untranslated regions. Although the functional consequences of these different 3'-end mRNAs are not known, they may show altered stability or cellular localization. In addition to the alternative splicing in the 3'-end of the SPEC1 mRNA, there is also a SPEC1 splice variant from within the SPEC1 coding sequence, SPEC1-β. SPEC1-β, was detected by RT-PCR as a minor transcript in a variety of human tissues. Interestingly, the mechanism by which the SPEC1-β mRNA transcript is produced involves intron retention. Intron retention occurs in other genes and frequently occurs in cancer. For instance, there is an aberrant inclusion of intron 9 in CD44 transcripts in tumor tissues, including bladder cancer, breast cancer, ovarian cancer, and gastrointestinal tumors (Matsumura et al., 1995; Yoshida et al., 1995; Bolodeoku et al., 1996). Although SPEC1-β is present at low levels in a variety of normal tissues, it is possible that this isoform may occur more frequently in tumor tissues. Experiments using *in vitro* translation confirmed that the SPEC1-β cDNA generates the expected 3.8 kDa protein product. Since the SPEC1-β protein lacks a CRIB domain involved in Cdc42 binding and contains a potential membrane targeting sequence, it may function as a signaling molecule independent of Cdc42 control. Taken together, these results suggest the possibility that the SPEC1-β protein may exist *in vivo* and may have its own unique biological activities. An additional potentially exciting result is that SPEC1 mRNA expression appears to correlate with metastatic aggressiveness, where more highly aggressive cell lines apparently lose SPEC1 mRNA expression. In preliminary experiments examining the protein levels of SPEC1 in a panel of breast cancer cell lines of increasing metastatic aggressiveness, there appeared to be a variable expression of SPEC1 protein (data not shown), however these results await future validation.

In addition to the originally proposed Specific Aims, we have also begun to address other interesting aspects of SPEC1 biology. As a research on this project has progressed, we have made several intriguing discoveries that we have started pursuing. Furthermore, we have continued to develop useful reagents to investigate SPEC1 biology including polyclonal antibodies generated against SPEC1. These antibodies are suitable for Western blotting and are also able to detect SPEC1 by immunofluorescence in SPEC1 overexpressing cells. One interesting aspect of SPEC1 biology that we have investigated is the potential for the SPEC proteins to interact with phospholipids in a manner similar to other known Cdc42 binding proteins including the WASP family.

Using *in vitro* binding assays with recombinant SPEC1 protein, we have shown that SPEC1 strongly bound phosphatidylinositol (PtdIns)-3-P, PtdIns-4-P, PtdIns-5-P, phosphatidic acid, and PtdIns-(3,5)P_2, weakly bound PtdIns –(3,4)P_2, PtdIns-(4,5)P_2 and PtdIns-(3,4,5)P_3, and did not bind phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphaditylinositol. These results are interesting because they begin to define at a molecular level the potential regulation of the SPEC family of proteins by phospholipids.

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SPECs, Small Binding Proteins for Cdc42*

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The Rho GTPase, Cdc42, regulates a wide variety of cellular activities including actin polymerization, focal complex assembly, and kinase signaling. We have identified a new family of very small Cdc42-binding proteins, designated SPECs (for Small Protein Effector of Cdc42), that modulates these regulatory activities. The two human members, SPEC1 and SPEC2, encode proteins of 79 and 84 amino acids, respectively. Both contain a conserved N-terminal region and a centrally located CRIB (Cdc42/Rac Interactive Binding) domain. Using a yeast two-hybrid system, we found that both SPECs interact strongly with Cdc42, weakly with Rac1, and not at all with RhoA. Transfection analysis revealed that SPEC1 inhibited Cdc42-induced c-Jun N-terminal kinase (JNK) activation in COS1 cells in a manner that required an intact CRIB domain. Immunofluorescence experiments in NIH-3T3 fibroblasts demonstrated that both SPEC1 and SPEC2 showed a cortical localization and induced the formation of cell surface membrane blebs, which was not dependent on Cdc42 activity. Cotransfection experiments demonstrated that SPEC1 altered Cdc42-induced cell shape changes both in COS1 cells and in NIH-3T3 fibroblasts and that this alteration required an intact CRIB domain. These results suggest that SPECs act as novel scaffold molecules to coordinate and/or mediate Cdc42 signaling activities.

The Rho GTPase, Cdc42, regulates numerous and diverse biological activities at both the biochemical and cellular levels. Cdc42 influences membrane trafficking (1), cytokinesis (2), and kinase signaling pathways, leading to transcriptional activation (3). Cdc42 is best known for controlling the formation of filopodia, thin actin-containing structures that protrude from the cell surface (4, 5). In addition to the direct effect of Cdc42 on filopodia formation, Cdc42 can also induce cross-activation of Rac, leading to membrane ruffling (5). This cross-talk among small GTPases may function to coordinate the formation and dismantling of different cortical actin structures, such as filopodia, ruffles, lamellopodia, and membrane blebs, often seen during cell migration (6).

The ability of Cdc42 to influence these diverse activities stems from its interactions with a large number of different kinase and non-kinase effector proteins. Although GTP-bound Cdc42 usually interacts with downstream effector proteins containing the conserved binding motif called a CRIB[1] domain (7), some downstream Cdc42 effector proteins such as IQGAP do not contain CRIB domains (8, 9). To date, six distinct families of CRIB domain-containing Cdc42 effector proteins have been identified: PAK (10, 11), MRCK (12), ACK (13), MLK (7, 14), WASP (15, 16) and MSE55/BORG/CEP (17, 18). The first four of these families are kinases. The most extensively studied Cdc42 effector proteins, the PAK kinases (3), participate in the Cdc42-mediated cytoskeleton rearrangements that lead to cell motility (19). PAK kinases also activate the JNK and p38 stress kinase pathways (20–22) and are targets of caspase-mediated proteolytic cleavage during apoptosis (23). MRCK kinases affect actin/myosin reorganization by phosphorylating non-muscle myosin light chain (12). Less is known about the signaling pathways and cellular processes affected by ACK tyrosine kinases, but they may influence cell adhesion signals (24). The fourth family, MLK kinases, play a role in kinesin function and JNK activation (14, 25, 26).

WASP and a related protein, N-WASP, comprise a family of non-kinase CRIB-containing proteins that function in actin polymerization (15, 16). N-WASP regulates filopodia formation by producing free actin filaments either via its cofilin actin-severing domain (16) or through interactions with the actin-severing protein, profilin (27). Both WASP and N-WASP also positively regulate the ARP2/3 protein complex, which stimulates actin nucleation (28, 29). The other non-kinase, CRIB-containing set of Cdc42 effector proteins, is the MSE55/CEP/BORG family, which is the most structurally diverse. This family, consisting of five members, all induce long actin-containing cellular extensions in NIH-3T3 fibroblasts (17, 18).

With so many different Cdc42 effector proteins, many of which may coexist in a single cell, competition and/or some mechanism for coordination must exist to ensure that the proper Cdc42 signal is propagated. Although many individual Cdc42 effector proteins have been studied, little is known about how these effector proteins cooperate and/or compete with each other, either in regulating the cytoskeleton or in kinase signaling. Here, we have identified a novel family of Cdc42 effector proteins that may play a role in this higher level of coordination. This new family, designated SPEC (for Small Protein Effector of Cdc42) has two human members, SPEC1 and SPEC2. Both are very small and are highly conserved. SPECs appear to have multifaceted activities, of which some are independent of Cdc42 binding and some are dependent on Cdc42

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF187845 and AF189692.

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[1] The abbreviations used are: CRIB, Cdc42/Rac interactive binding domain; AKAP, A-kinase anchoring protein; EST, expressed sequence tag; JNK, c-Jun N terminal kinase; SPEC, small protein effector of Cdc42; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; contig, group of overlapping clones; HA, hemagglutinin.

binding. For instance, expression of SPECs in NIH-3T3 fibroblasts induced membrane blebbing. SPEC-induced blebbing did not require Cdc42 activity because blebbing still occurred with a CRIB domain mutant of SPEC or in the presence of dominant negative Cdc42. SPEC1 blocked both Cdc42-induced JNK activity and altered Cdc42-induced morphology changes in COS1 cells and in NIH-3T3 fibroblasts in a manner that required an intact CRIB domain and, thus, was dependent on Cdc42 binding. Together these results suggest that SPECs act as novel scaffold molecules to coordinate and/or mediate Cdc42 signaling activities.

EXPERIMENTAL PROCEDURES

Identification and Cloning of SPECs—Clones of SPEC1 were identified from a TBLASTN search of the expressed sequence tag (EST) data base at the National Center for Biotechnology Information (NCBI) using the 16-amino acid CRIB core sequence of MSE55 (7) (ISHPLGDFRHTMHVGR) as a query. Several of these human EST clones were obtained from the I.M.A.G.E. consortium (clone ID numbers: 257442, 139233, 160147) and sequenced on an Applied Biosystem 377 DNA sequencer. The GenBank℠ accession number of SPEC1 is AF187845. Furthermore, EST clones for human and mouse SPEC1 are quite abundant and are represented by the NCBI Unigene identifiers Hs.22065 and Mm.28189, respectively.

Additional TBLASTN searches of the non-redundant nucleotide data base using the amino acid sequence of SPEC1 as query identified three well separated DNA sequences that if transcribed as a single mRNA and properly spliced might encode a second SPEC-like protein. Two adapter-primers, 5′-GAGGGATCCAGTGAATTCTGGTTGTGT-3′ and 5′-GAGCTCGAGCTATCCCGCCTTCGTATC-3′, derived from these genomic sequences and corresponding to each end of the putative coding sequence, were synthesized. These two primers were used in reverse transcription-PCR with MCF-7 breast cancer cell RNA as template. After PCR, an approximately 250-base pair PCR fragment was obtained, cut with *Bam*HI/*Xho*I, and subcloned into the *Bam*HI-*Xho*I site of pCAF2, a mammalian expression vector (17). DNA sequencing revealed that the nucleotide sequence of this PCR product was, as expected, derived from the three different 5q31 genomic fragments and encodes a protein, designated SPEC2, that was quite similar to SPEC1. SPEC2 has GenBank℠ accession number AF189692.

Yeast Two-hybrid Assays—The yeast two-hybrid assay was performed in the Y190 yeast strain using the pYTH GAL4 DNA binding domain yeast vector and pACT-II GAL4 activation domain yeast vectors (30). cDNAs for SPEC1 (amino acids 2–79) or SPEC2 (amino acids 2–84) were subcloned downstream of the GAL4 DNA binding domain in pYTH-9, and integrated strains were generated as described (30). Deletion mutants of SPEC1 consisting of amino acids 2–27 (SPEC1-del1) or amino acids 48–79 (SPEC1-del2) were constructed in the same way. Wild type and activated mutants of Cdc42, Rac, and Rho were subcloned downstream of the GAL4 activation domain in pACT-II. Protein-protein interactions were detected by assaying for β-galactosidase activity in a filter assay or by growth on nutrient agar plates lacking histidine (30).

Mammalian Expression Vectors for SPEC1, SPEC2, and Mutants—The coding sequence of SPEC1 was amplified by PCR from the I.M.A.G.E. cDNA clone (ID 22978) using the two primers 5′-GAGGGATCCAGTGAATTTTGGCACAAAC-3′ and 5′-GAGCTCGAGCTATAAGCCCCTAGAATTG-3′. This PCR product was then subcloned in-frame into the *Bam*HI-*Xho*I sites downstream of an N-terminal myc epitope-tagged pcDNA-III (Invitrogen) or an N-terminal FLAG epitope-tagged pCAF2 mammalian expression vector. An additional C-terminal myc epitope-tagged SPEC1 construct was constructed in the pcDNA-III mammalian expression vector. Untagged SPEC1 constructs were also constructed using the bicistronic enhanced green fluorescent protein (EGFP) vector (CLONTECH). The integrity of all constructs was confirmed by DNA sequencing.

Several SPEC1 mutant constructs were generated from the epitope-tagged constructs by using SPEC1 sequence-specific oligonucleotides and the QuickChange mutagenesis kit (Stratagene). Four single or multiple SPEC1 point mutants containing alanine substitutions in the N terminus (SPEC1-C10A,C11A), the CRIB domain (SPEC1-H38A and SPEC1-P35A,H38A,H41A) or in the C terminus (SPEC1-Q62A,K64A) were constructed. The integrity of all constructs was confirmed by DNA sequencing. All of these mutants and their parent constructs had approximately similar levels of expression in transfected cells as judged by immunofluorescence.

JNK Kinase Assays—COS1 cells were used to examine whether SPEC influenced JNK kinase activation using ATF-2 as substrate as described (31). Equal amounts of DNA were used in each transfection.

Immunofluorescence—Immunofluorescence of NIH-3T3 fibroblasts was performed essentially as described (17) except that LipofectAMINE Plus (Life Technologies, Inc.) was used as the transfection reagent. Twenty-four hours post-transfection, NIH-3T3 fibroblasts were fixed and permeabilized on glass coverslips coated with polylysine. Coverslips were stained with the myc and FLAG anti-epitope primary antibodies including mouse anti-FLAG® M2 (Sigma), mouse anti-c-myc monoclonal antibody (Sigma), and polyclonal anti-FLAG®/Octaprobe antibody (Santa Cruz Biotechnology). Fluorescein-conjugated goat anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA), Texas Red anti-mouse (Jackson ImmunoResearch Laboratories, Inc.), and FITC-conjugated goat-anti-rabbit (Rockland Immunochemicals) were used as secondary antibodies. Texas Red-conjugated Phalloidin (Sigma) was used to stain F-actin. Nuclear and phosphotidylserine staining was also performed using 4,6-diamidino-2-phenylindole (Sigma) and annexin-V (Trevigen, Inc.), respectively.

Blebbing cells were quantified in vector alone and in SPEC-ransfected cells 24 h post-ransfection. Cells with two or more outpouchings were scored as positive for membrane blebbing. Fifty to one hundred cells were scored from each of at least three independent transfections. A Zeiss Photomicroscope III equipped with a Planapo 63X/1.4 NA phase 3 objective was used, and photographs were taken with Kodak TMAX400 film using a Nikon N6006 camera.

RESULTS

Identification of Genes for SPEC1 and SPEC2—Most known Cdc42-inding proteins contain a conserved core domain, the CRIB domain (7). We looked for additional proteins that might bind Cdc42 by searching the human EST data base for sequences similar to the CRIB domain of the non-kinase Cdc42 effector protein MSE55/CEP1 (17) and identified many cDNAs encoding the same small protein. The DNA sequences of several independent human clones comprising a contig spanning 1.8 kilobases each showed an open reading frame of 79 amino acid residues, which we designated SPEC1 (Fig. 1). These cDNAs encoding the SPEC1 protein contained a Kozak consensus sequence and an upstream in-frame stop codon and did not encode any proteins longer than SPEC1. A second human SPEC family member, designated SPEC2, was identified by searching the non-redundant GenBank™ data base. This search identified three short separated genomic regions from chromosome 5q31, spanning at least 28 kilobases (GenBank™ accession numbers AC001489 and AC001223) that if transcribed and properly spliced would encode a second SPEC-like protein. To clone the SPEC2 cDNA sequence, primers were designed for the two extreme ends of the genomic regions and used in reverse transcription-PCR. This approach yielded a 250-base pair PCR fragment containing an open reading frame of 84 amino acids (Fig. 1). Comparison of this sequence with 5q31 genomic sequences confirmed that the full-length SPEC2 protein was encoded in three exons spanning at least 28 kilobases.

In addition to the human SPEC1 and SPEC2 proteins, we have also identified both SPEC1 and SPEC2 homologs in other organisms. We have identified SPEC1 homologs from mouse (GenBank™ AI472516) and chicken (GenBank™ AI981286) that are 96 and 83% identical to SPEC1 at the amino acid level, respectively (Fig. 1). We have also identified SPEC2 homologs from mouse (GenBank™ AW061198), rat (GenBank™ AA944330), *Drosophila* (AA820736), and ascidian (*Halocynthia roretzi*; GenBank™ AV382466) (Fig. 1). Identification of the SPEC proteins in such diverse organisms suggests their function may be conserved through evolution.

SPECs Represent a Novel Family of Cdc42-binding Proteins—SPEC1 and SPEC2 represent two members of a new protein family that are 76% similar over their entire length (Fig. 1) and encode proteins with predicted molecular masses of 7.9 kDa and 8.4 kDa, respectively. Both SPECs contain a

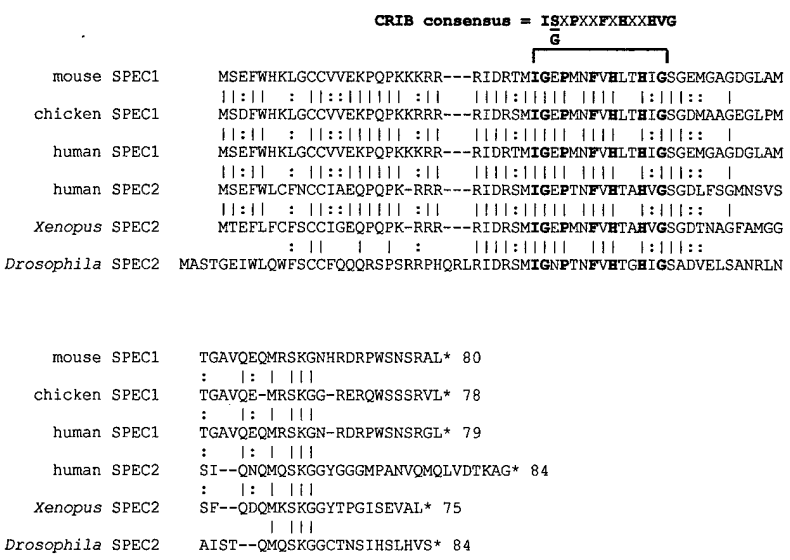

FIG. 1. **The SPEC protein family.** Alignment of full-length human SPEC1 and SPEC2 amino acid sequences. ESTs corresponding to mouse SPEC1 (AI472516), chicken SPEC1 (AI981286), and *Xenopus* SPEC2 (AW644132) and *Drosophila* SPEC2 (AAF51990) are also shown. *Vertical lines* identify identical residues, *colons* represent conservative substitutions, and the *numbers* at the ends represent the total number of amino acid residues. The CRIB consensus is shown (7), and sequences matching this consensus sequence are shown in *bold letters*. Human SPEC1 and SPEC2 sequences are available from GenBank™ under accession numbers AF187845 and AF189692, respectively.

highly conserved N-terminal region and a typical CRIB domain. The CRIB domains of the SPECs extend beyond the CRIB core sequence and contain the consensus sequence DR(S/T)M**IGEPXNFVHXXHAG**SGD/E*AXX*G, where *A* represents an aliphatic amino acid, and bold letters identify the CRIB core (Fig. 1). In the C terminus of both proteins there is a relatively small conserved sequence containing the nine-amino acid consensus sequence, (V/I)Q(E/N)QM(R/Q)SKG (Fig. 1). This region may be part of an extended high affinity Cdc42 binding site (32, 33).

CRIB-dependent Cdc42 Binding by SPECs—Since proteins containing a consensus CRIB domain will bind Cdc42 and/or Rac (7), we predicted that both SPEC1 and SPEC2 also would interact with Cdc42 and/or Rac. We tested this prediction in a yeast two-hybrid system. Both SPEC1 and SPEC2 interacted strongly with a constitutively activated mutant of Cdc42 (Cdc42-Q61L), weakly with an activated mutant of Rac1 (Rac1-Q61L), and not at all with an activated mutant of RhoA (RhoA-Q63L) using both the β-galactosidase filter assay (Fig. 2) and growth on selective media (data not shown). Although interaction of SPECs with wild type Cdc42 and wild type Rac1 was not observed, our results are consistent with yeast two-hybrid experiments using other CRIB-containing Cdc42 effector proteins, such as WASP (30). Additionally, SPEC CRIB deletion mutants retaining either the N terminus (SPEC1-del1; amino acids 2–27) or the C terminus (SPEC1-del2; amino acids 48–79) were unable to bind to an activated Cdc42 mutant (Fig. 2). Collectively, these results demonstrate that SPECs can interact with Cdc42 in a CRIB-dependent fashion and suggest that SPECs may function normally in Cdc42-dependent signaling.

SPEC1 Expression Inhibits Cdc42-induced JNK Activity—Since a variety of studies have shown both that Cdc42 (31, 34) and some Cdc42 effector proteins (14, 20–22, 26) can activate JNK activity, we tested SPEC1 and several SPEC1 mutants for their effect on Cdc42-induced JNK activation. First, an expression construct of human SPEC1 carrying an N-terminal FLAG epitope tag was transfected into NIH-3T3 fibroblasts, and its expression was analyzed by Western blotting using a monoclonal antibody against the N-terminal FLAG epitope tag. Using this approach, SPEC1 was detected in lysates as an ~8 kDa species (data not shown), of which about 1 kilobase is contributed by the epitope tag. Second, several SPEC1 mutants were constructed, including two CRIB mutants and a C-terminal double mutant. The two CRIB mutants, SPEC1-H38A and SPEC1-P33A,H38A,H41A, contain alanine substitutions within critical contact sites known to be involved in Cdc42 binding (33, 34). The third mutant, SPEC1-Q62A,K66A, contained mutations within the nine-amino acid region conserved between both SPEC proteins that might be part of an extended high affinity Cdc42 binding site. COS1 cells were cotransfected with the expression vectors for GFP (control) or Cdc42-Q61L, hemagglutinin (HA) epitope-tagged JNK, and FLAG-tagged SPEC1 constructs. After 24 h, transiently expressed HA-JNK was isolated by immunoprecipitation and used for *in vitro* kinase assays. All SPEC1 constructs tested were not able to stimulate JNK activity on their own (Fig. 3*A*). Expression of Cdc42 with HA-JNK stimulated kinase activity (Fig. 3, *A* and *B*). Cotransfection of cells with wild-type SPEC1 significantly reduced the Cdc42-induced JNK activation (Fig. 3, *A* and *B*). In contrast, SPEC1-P33A,H38A,H41A or SPEC1-Q62A,K66A were markedly less effective at blocking Cdc42-induced JNK activation (Fig. 3, *A* and *B*). In addition, a similar failure to block Cdc42-induced JNK activation was also observed with the single CRIB domain mutant (data not shown). Although we cannot rule out the possibility that our overexpression studies have resulted in abnormally high levels of SPEC1, which may nonspecifically inhibit Cdc42 signaling pathways, these results may also suggest that SPEC1 modulates JNK activity by binding or sequestering Cdc42 through a CRIB-dependent interaction.

SPEC1 Expression Inhibits Cdc42-induced Morphological Changes in COS1 Cells—We next examined the cellular distribution of epitope-tagged SPEC1 expression by immunofluorescence. In COS1 cells, SPEC1 showed diffuse cytoplasmic localization (Fig. 4*B*). Additionally, SPEC-expressing cells did not show altered actin structures or cell morphology (data not shown). We next determined whether SPEC1 influenced Cdc42 function when co-expressed with Cdc42. COS1 cells expressing the constitutively active Cdc42-Q61L mutant exhibited a widely spread and flattened phenotype with some filopodia (Fig. 4, *A* and *B*). Cotransfection of SPEC1 with Cdc42-Q61L resulted in cells that were more elevated and much less spread than cells expressing Cdc42-Q61L alone (compare Fig. 4, *C* and *D* with *A*) or untransfected cells (data not shown). In contrast to what was seen with wild-type SPEC1, cells coexpressing Cdc42-Q61L and the CRIB domain mutant (SPEC1-H38A) resembled cells transfected with Cdc42-Q61L alone (compare Fig. 4, *E* and *F* with *A*). These results suggest that SPEC1 modifies Cdc42 function. In these experiments, SPEC1 appears to alter Cdc42 activity by binding to it via the CRIB domain. These results suggest that SPECs may function to block the interaction of Cdc42 with other effector proteins, although we cannot

	Cdc42	Cdc42-Q61L	Rac1	Rac1-Q61L	RhoA-Q63L
SPEC1	-	+++	-	+	-
SPEC2	-	+++	-	+	-
SPEC1-del1	ND	-	ND	-	-
SPEC1-del2	ND	-	ND	-	-

FIG. 2. **SPEC1 and SPEC2 interact with Cdc42 in yeast two-hybrid assays.** pYTH9 GAL4-DNA binding constructs were generated for SPEC2, SPEC1, or deletions of SPEC1 and then integrated into Y190 yeast cells. These yeast strains were used as host cells for transformations with pACT-GAL4 activation constructs to test interactions with Rho GTPases. The strength of the interaction was classified by the time taken for colonies to turn blue in the β-galactosidase filter assay: +++, <25 min; ++, 25–50 min; +50–100 min; −, no color change by 100 min; and *ND*, not determined.

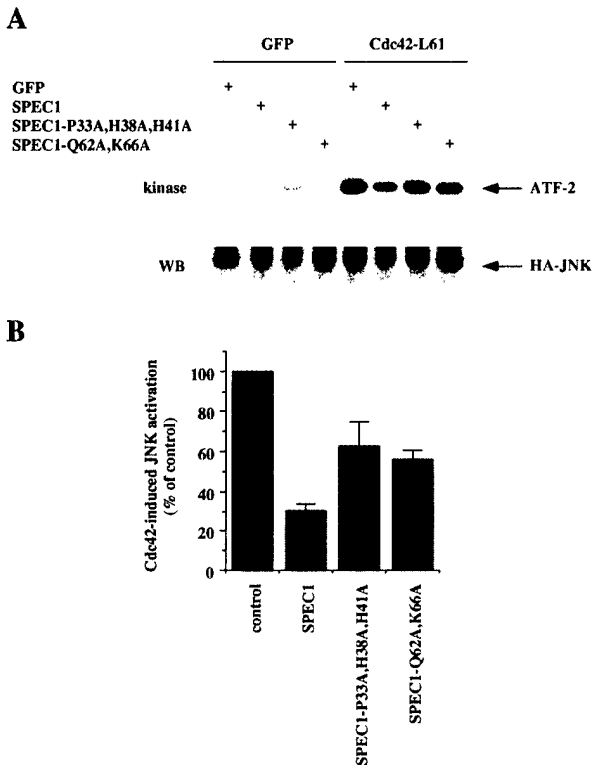

FIG. 3. **SPEC1 inhibits Cdc42-induced JNK activation.** COS1 cells were transfected with expression vectors for GFP (control) or activated Cdc42 mutant (Cdc42-QL61), HA-tagged JNK, and different FLAG-tagged SPEC1 constructs. Following immunoprecipitation, JNK activity was assayed using ATF-2 as substrate (*A*). Similar results were obtained in three independent experiments. This assay was normalized by Western blot analysis using anti-HA immunoprecipitates from the cellular lysates and immunodetected with JNK antisera as described under "Experimental Procedures." Results are the averages ± S.E. of three experiments. *WT*, wild type.

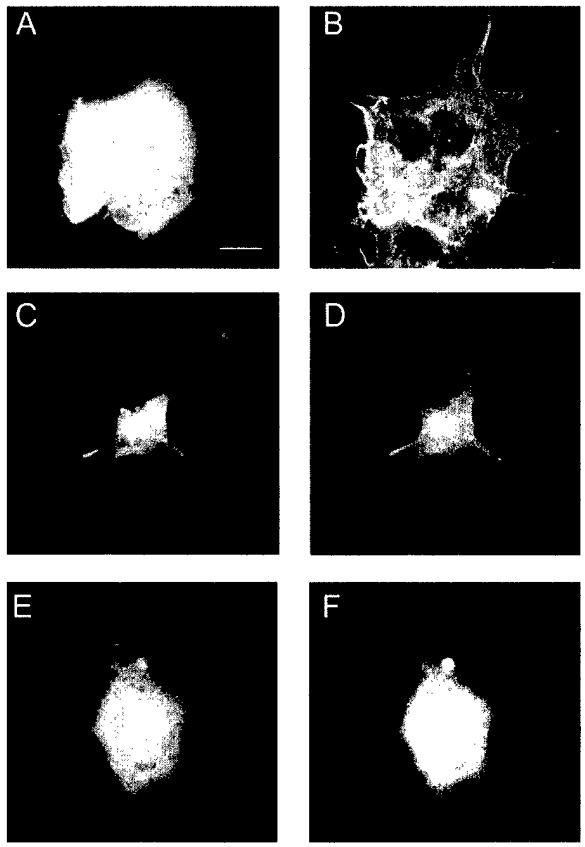

FIG. 4. **SPEC1 alters Cdc42 activity in COS1 cells.** COS1 cells were transfected with myc-tagged Cdc42-Q61L and processed for indirect immunofluorescence at 24 h post-transfection using monoclonal antibodies specific for the myc epitope tag followed by either Texas Red-conjugated goat anti-mouse antibodies or FITC-conjugated goat anti-mouse antibodies (*A*, *C*, and *E*). Cells transfected with myc-tagged Cdc42-Q61L alone were co-stained for F-actin (*B*). Cells cotransfected with either FLAG-tagged wild-type SPEC1 (*D*) or FLAG-tagged SPEC1-H38A (*F*) were processed for indirect immunofluorescence using a polyclonal FLAG™/octaprobe antibody followed by FITC-labeled goat anti-rabbit secondary antibodies. The morphology of the COS1 cells transfected with Cdc42-Q61L resemble those cotransfected with the SPEC1-H38A mutant (compare *A* with *E*). *Bar*, 10 μm.

rule out the possibility that the observed blocking activity was due to overexpression of SPEC1 protein.

SPEC Expression Induces Non-apoptotic Blebbing in NIH-3T3 Fibroblasts—Since SPEC1 expression did not noticeably alter the morphology of COS1 cells, we studied the effects of SPEC1 expression in NIH-3T3 fibroblasts. In NIH-3T3 fibroblasts, SPEC1 displayed a predominant cortical localization (Fig. 5*A*), and frequently, these transfected cells showed extensive membrane blebbing (Fig. 5*A*). F-actin stained strongly within the periphery of the blebs but not within the blebs (Fig. 5*B*). Expression of SPEC2 also showed the same cortical localization, membrane blebbing, and F-actin staining phenotype (Fig. 5, *C* and *D*). A similar pattern of cortical staining and blebbing were observed with a myc epitope tag located either at the N or C terminus of SPEC1 and using a 20-fold range of plasmid concentrations (100 ng to 2 μg; data not shown). Although this SPEC-induced membrane blebbing is morphologically similar to the membrane blebbing associated with apoptosis, there is no functional association of the SPEC-induced blebbing with apoptosis. That is, neither nuclear condensation following 4,6-diamidino-2-phenylindole staining of nuclei nor annexin-V positive staining, a marker for phosphotidylserine flipping in the membrane, was observed in these transfected cells (data not shown).

Quantitatively, membrane blebbing was observed in 40–60% of the FLAG epitope-tagged SPEC1 transfected cells but only in about 5% of cells expressing the vector-alone control (Fig. 6). We also used a bicistronic expression vector expressing both SPEC1 and EGFP from the same vector to rule out the

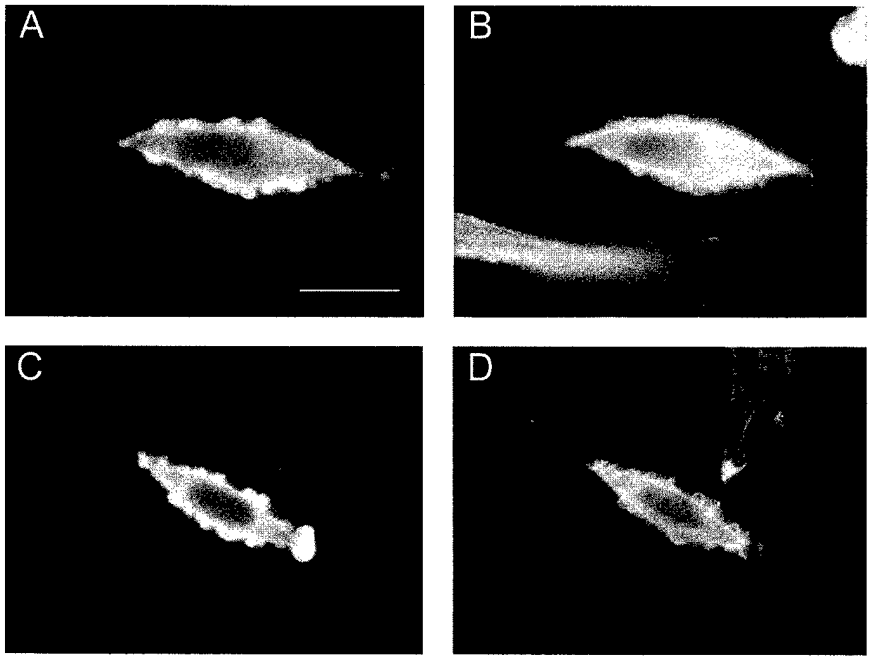

FIG. 5. **SPEC1 and SPEC2 induce membrane blebbing in NIH-3T3 fibroblasts.** N-terminal FLAG epitope-tagged SPEC1 (*A* and *B*) or N-terminal FLAG epitope-tagged SPEC2 (*C* and *D*) constructs were transfected into NIH-3T3 fibroblasts. The cells were fixed 24 h after transfection and processed for indirect immunofluorescence using an anti-FLAG monoclonal antibody followed by FITC-conjugated goat anti-mouse secondary antibody to detect SPEC1(*A*) and SPEC2 (*C*) protein expression. Cells were co-stained for F-actin using Texas Red conjugated-phalloidin (*B* and *D*). *Bar*, 10 μm.

possibility that the epitope tags might influence SPEC function. About 40% of the cells expressing the bicistronic SPEC1 construct showed a blebbing phenotype, whereas only 10% were blebbing with the EGFP-alone vector (Fig. 6). Taken together, these results demonstrate that expression of SPECs, whether epitope-tagged or untagged, leads to membrane blebbing in NIH-3T3 fibroblasts.

SPEC1-induced Blebbing Is Independent of Cdc42 Activity—To determine whether any of the three conserved regions in SPECs (see Fig. 1) are necessary for SPEC1-induced membrane blebbing, we examined the phenotype of cells transfected with various SPEC1 mutants. An additional N-terminal mutant, SPEC1-C10A,C11A was created within two conserved cysteine residues because of the potential role of these residues in lipid modification or protein interactions. Using cell counting it was found that both the positive and negative controls gave the expected results: approximately 44% of N-terminal FLAG-tagged SPEC1-transfected cells blebbed, as compared with only 4% using a vector-alone control (Fig. 7). The C-terminal double point mutant, SPEC1-Q62A,K66A, had no effect on the level of blebbing (Fig. 7). In contrast, the cells expressing the N-terminal mutant (SPEC1-C10A,C11A), which showed a similar level of expression and cortical localization, produced the blebbing phenotype in only 20% of the transfected cells (Fig. 7). Single or triple amino acid substitutions within the CRIB domain of SPEC1 resulted in somewhat fewer blebs, although they still produced significantly more than the negative controls (30% *versus* 4%; Fig. 7). Additional studies expressing a dominant negative mutant of Cdc42 (Cdc42-T17N) alone did not induce membrane blebbing, and co-expression with SPEC1 did not block membrane blebbing (data not shown). These results support a model whereby SPEC1-induced blebbing does not occur through classical Cdc42-effector interactions and suggest that SPEC1 may act independently of Cdc42 or perhaps upstream of Cdc42 to induce membrane blebbing. These data also confirm that SPEC-induced membrane changes are not directly due to sequestration of Cdc42.

SPEC1 Expression Alters Cdc42-induced Changes in Cellular Morphology in NIH-3T3 Fibroblasts—To more clearly define the relationship between Cdc42 activity and SPECs, we tested the effect of SPEC1/Cdc42 co-expression in NIH-3T3 fibroblasts. In these fibroblasts, expression of Cdc42L61 resulted in cells that predominantly exhibited a membrane ruffling phenotype, possibly through activation of Rac signaling. We then cotransfected SPEC1 or the SPEC1-CRIB mutants (SPEC1-H38A or SPEC1-P33A,H38A,H41A) with constitutively active Cdc42 and quantified by cell counting the number of transfected cells showing a ruffling phenotype. Expression of a constitutively active Cdc42 mutant (Cdc42-Q61L), but not wild type Cdc42 (data not shown), in NIH-3T3 fibroblasts induced marked membrane ruffling in 52% of the transfected cells (Fig. 8, *A* and *B*). Co-transfection of SPEC1 blocked ruffling in all but 5% of the transfected cells and increased the number of blebbing cells (Fig. 8, *C* and *D*). Coexpression of the SPEC-H38A mutant resulted in 34% of the cells showing a membrane ruffling phenotype (Fig. 8, *E* and *F*) and resembled cells transfected with Cdc42 alone (compare Fig. 8, *A* and *B*, with *E* and *F*). Similar results were also obtained with the

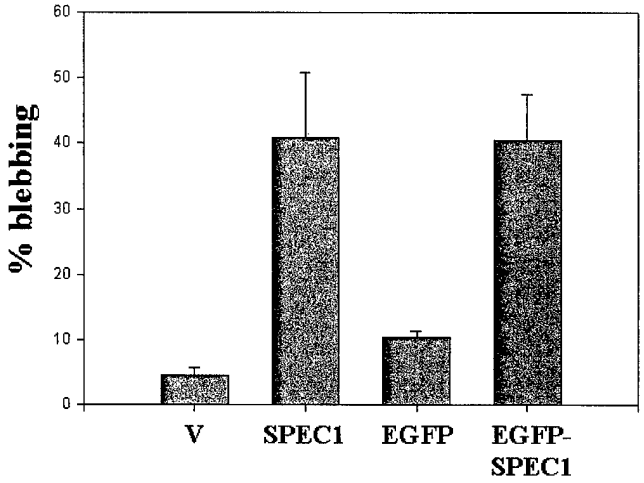

FIG. 6. **Quantitative analysis of the effect of tagged and untagged SPEC1 on blebbing.** NIH-3T3 fibroblasts were transfected, fixed, and processed for indirect immunofluorescence. NIH-3T3 fibroblasts showing at least two membrane blebs were scored as positive for blebbing as described under "Experimental Procedures"; values indicate mean and S.D. *V*, pCAF2 vector alone; *SPEC1*, pCAF2-SPEC1; *EGFP*, EGFP vector alone; *SPEC1-EGFP*, bicistronic SPEC1-EGFP vector.

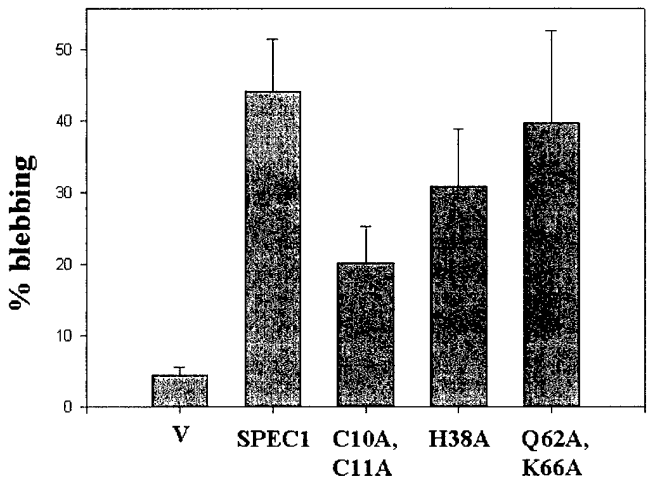

FIG. 7. **Activity of different SPEC1 mutants in inducing membrane blebbing.** Wild-type SPEC1 and different SPEC mutants were transfected into NIH-3T3 fibroblasts. At 24 h post-transfection, cells were fixed, processed for indirect immunofluorescence, and counted for blebbing as described above in the legend to Fig. 6; values indicate the mean and S.D. *V*, pCAF2 vector alone; *SPEC1*, pCAF2-SPEC1; *C10A,C11A*, pCAF2-SPEC1-C10A,C11A; *H38A*, pCAF2-SPEC1-H38A; *Q62A,Q66A*, pCAF2-SPEC1-Q62A,Q66A.

SPEC1-P33A,H38A,H41A CRIB mutant (data not shown). It is also worthy to note that in these cotransfections experiments, SPEC1 and Cdc42 proteins appear to localize to similar regions within the cells, suggesting that SPECs and Cdc42 may be contained within the same signaling complexes (Fig. 8). As with COS1 cells, these transfections demonstrate that SPEC1 expression led to an altered Cdc42-induced morphology and that this alteration is dependent on the presence of an intact CRIB domain.

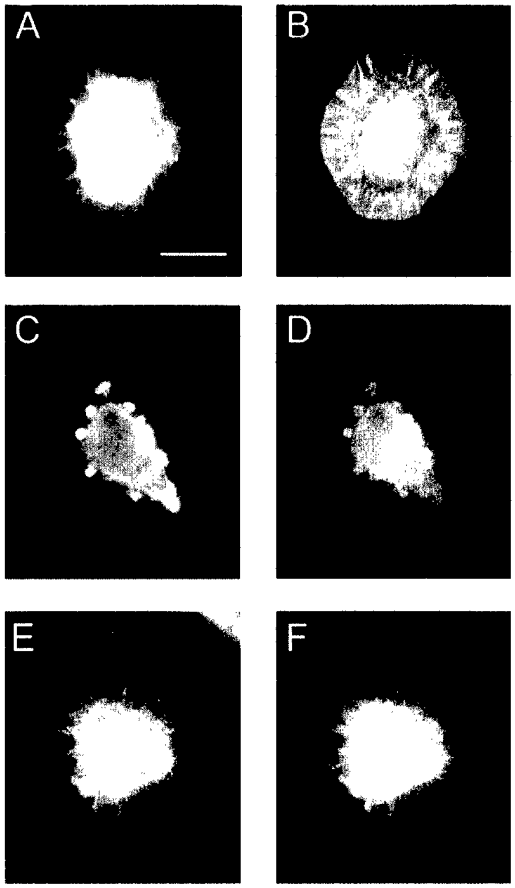

FIG. 8. **SPEC1 alters Cdc42-induced cell shape changes.** NIH-3T3 fibroblasts were transfected with myc-tagged Cdc42-Q61L and processed for indirect immunofluorescence at 24 h post-transfection using monoclonal antibodies specific for the myc epitope tag followed by either Texas Red-conjugated goat anti-mouse antibodies or FITC-conjugated goat anti-mouse antibodies (*A*, *C*, and *E*). Cells transfected with myc-tagged Cdc42-Q61L alone were co-stained for F-actin (*B*). Cells cotransfected with either FLAG-tagged wild type SPEC1 (*D*) or FLAG-tagged SPEC1-H38A (*F*) were processed for indirect immunofluorescence using a polyclonal FLAG™/octaprobe antibody followed by FITC-labeled goat anti-rabbit secondary antibodies. The morphology of the NIH-3T3 fibroblasts transfected with Cdc42-Q61L resemble those co-transfected with the SPEC1- CRIB mutant (compare *A* with *E*). *Bar*, 10 μm.

DISCUSSION

Here we identify a new family of proteins capable of binding to Cdc42, designated SPECs, found in many eukaryotic species. The two human members, SPEC1 and SPEC2, are the smallest known GTPase-binding proteins. Their small size may explain why they were not detected in previous biochemical screens based on binding to Cdc42. Overexpression of different combinations of SPECs, SPEC mutants, and Cdc42 showed that SPEC expression inhibited Cdc42-induced JNK activity. SPEC overexpression also altered or reversed the cellular morphologies produced when Cdc42 is overexpressed in COS1 cells and in NIH-3T3 fibroblasts. The membrane blebbing induced by SPEC overexpression in NIH-3T3 fibroblasts was not observed in COS1 cells, possibly due to quantitative differences in expression levels of SPEC proteins between the two cell types. Nevertheless, these results show that SPECs are capable of modifying Cdc42-dependent signaling at both the biochemical and cellular levels in a CRIB-dependent manner. SPEC binding could prevent the interaction of Cdc42 with other effector proteins. Consistent with this model, a polypeptide containing just the CRIB domain of PAK can effectively inhibit Cdc42 activation of JNK kinase (34) and block transcriptional activation (35), whereas a polypeptide containing the CRIB domain of ACK-1 can act as a Cdc42-specific inhibitor, blocking v-Ha-Ras-induced transformation (36).

However, we do not know if the specific biochemical and biological effects observed here with overexpressed SPECs reflect the normal function of these small proteins. In particular, SPEC overexpression induced membrane blebbing in NIH-3T3 fibroblast that was not blocked by dominant negative Cdc42 expression. Despite these findings, it is still possible that SPECs function in Cdc42-induced morphological changes, since a dominant negative approach may not rescue the abnormal morphology of overexpressed SPEC protein. Furthermore, various studies have shown that non-apoptotic membrane blebs function normally in cell spreading (37, 38) and locomotion (39–41). Mechanistically, membrane blebs occur at sites where the cortical actin is locally depolymerized or detached from the membrane (38, 40, 41) via alteration in cortical actin-binding proteins (40), myosin light chain kinase activity (42, 43), and/or focal complex assembly (43). Thus, it is tempting to speculate that SPECs may function as classical Cdc42 effector proteins by altering the normal signaling pathways leading to actin, myosin, and/or focal complex assembly.

The existence of small proteins that bind important signaling molecules is not unique to Cdc42. Recently, an 18-kDa protein, A-kinase anchoring protein-18 (AKAP18), was found to function as a scaffold protein, coupling protein kinase A signaling to calcium and sodium channels (44–46). Interestingly, AKAP18 and SPECs share many structural and functional similarities. First, both are small proteins: AKAP18, SPEC1, and SPEC2 are 81, 79, and 84 amino acids long, respectively. Second, both bind their ligands, protein kinase A or Cdc42, through their central binding regions. Third, both localize to the plasma

membrane. Although the membrane localization of AKAP18 involves lipid modification of the N terminus, we have not yet identified the region required to target SPECs to the membrane in NIH-3T3 fibroblasts. Based on these similarities, we speculate that SPECs, like AKAP18, may function as scaffolding molecules to recruit other signaling proteins to Cdc42 complexes. Future studies are aimed at identifying such SPEC-binding partners.

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The genomic structure of the human SPEC1 gene reveals complex splicing and close promoter proximity to the AF1q translocation gene

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Abstract

SPECs are small Cdc42 signaling molecules. In mammals, two genes, SPEC1 and SPEC2, encode proteins of 79 and 84 amino acid residues, respectively. Here we report the expression and genomic organization of the human SPEC1 gene. Using Northern blot analysis, three major SPEC1 mRNA transcripts of 1.6, 3.3, and 6.3 kb were detected. Identification and sequencing of different sized SPEC1 cDNA clones revealed that the transcript size heterogeneity was due to alternative splicing in the 3′-untranslated region. In addition, a distinct SPEC1 splice variant from within the coding sequence, SPEC1-β, was identified and detected in a variety of human tissues. Analysis of the genomic organization of SPEC1 revealed that the coding sequence of the SPEC1 isoform was derived from exons 2, 3 and 4, while the SPEC1-β isoform was derived from exon 2 and a read-through event of intron 2. Examination of the 5′-end of the SPEC1 genomic sequence revealed that AF1q, a previously identified gene involved in translocations with the MLL (mixed-lineage leukemia) gene, was 631 bp away in a head-to-head orientation. This intergenic sequence containing the putative promoter region for both SPEC1 and AF1q genes did not contain a TATA box or CAAT box. Transfection experiments using an AF1q promoter luciferase reporter construct in a variety of cells including Cos1 cells, Jurkat T-cells, MCF-7 breast cancer cells, and NIH-3T3 fibroblasts showed no promoter activity. In contrast, a SPEC1 promoter luciferase reporter construct showed high levels of reporter activity in Cos1 and MCF-7 cells, low activity in NIH-3T3 fibroblasts and no activity in Jurkat T-cells. These promoter analyses suggest that although SPEC1 and AF1q genes share the same promoter region, they are not coordinately regulated.

Keywords: Alternative splicing; Cdc42; Gene expression; Promoter region; Translocation

1. Introduction

The Cdc42 GTPase is a signaling molecule that regulates a diverse array of biological processes (Johnson, 1999). Most prominently, Cdc42 regulates actin polymerization leading to the formation of filopodia and other actin-containing structures (Kozma et al., 1995; Nobes and Hall, 1995). In addition to these cytoskeletal effects, Cdc42 can also regulate other biological processes such as cytokinesis (Dutartre et al., 1996; Drechsel et al., 1997), cell polarity (Kroschewski et al., 1999) and a variety of kinase signaling pathways leading to transcriptional activation (for review, see Johnson, 1999). A large and diverse set of molecules has been identified that interact with Cdc42 and function as downstream effector proteins. A common feature of many of these Cdc42 effector proteins is the presence of a CRIB/GBD domain involved in binding Cdc42 (Burbelo et al., 1995; Pirone et al., 2001). To date, nine distinct families of human CRIB domain-containing Cdc42 effector proteins derived from 25 genes have been identified: ACK (Manser et al., 1993), PAK (Manser et al., 1994), MLK (Burbelo et al., 1995; Teramoto et al., 1996), WASP (Symons et al., 1996; Miki et al., 1998), MRCK (Leung et al., 1998), MSE55/CEP/BORG (Burbelo et al., 1999; Joberty et al., 1999; Hirsch et al., 2001), Gene 33 (Makkinje et al., 2000), PAR-6 (Qiu et al., 2000) and SPECs (Pirone et al., 2000).

Of all the CRIB-containing Cdc42-binding proteins, the SPEC family members are the smallest in size (Pirone et al., 2000). In humans, two distinct genes, SPEC1 and SPEC2,

Abbreviations: CRIB, Cdc42/Rac interactive binding domain; EST, expressed sequence tag; MLL, mixed-lineage leukemia; RT-PCR, reverse transcription-polymerase chain reaction; SPEC, small protein effector of Cdc42

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encode proteins of 79 and 84 amino acid residues, respectively. Analysis of SPEC homologs from a number of additional species including *Drosophila*, chickens, and mice reveal that they are highly conserved and contain a central CRIB domain involved in Cdc42 binding. Transfection analysis showed that SPEC1 blocks Cdc42-induced JNK activity and Cdc42-induced morphological activities, suggesting a potential role in blocking Cdc42 activity. Although the exact biological function of these molecules is still not completely known, overexpression of SPEC1 and SPEC2 in NIH-3T3 fibroblasts induces the formation of cell surface membrane blebs, suggesting that they may normally function in focal complex assembly or cortical membrane integrity (Pirone et al., 2000).

In view of the biological activities and unusually small size of SPEC1, we have characterized its RNA distribution, genomic organization and promoter activity. We have determined that SPEC1 mRNA is ubiquitously expressed and has several different mRNA transcripts that arise though alternative splicing in the 3′-untranslated region. We have also identified a SPEC1 splice variant from within the coding sequence, SPEC1-β, and demonstrate using RT-PCR that it is ubiquitously expressed. Sequence comparison of the SPEC1 cDNA with a SPEC1-containing BAC genomic clone revealed that the gene was composed of six exons of which exons 2, 3, and 4 generate the coding sequence. By analyzing the 5′-end of the SPEC1 gene, we found that the AF1q gene is in a head-to-head orientation with the SPEC1 gene and is separated by a maximum distance of approximately 600 bp. Functional promoter analysis of this intergenic sequence revealed transcriptional activity for the SPEC1 gene, but not for the AF1q gene, suggesting that additional regulatory elements are required for AF1q transcriptional activity.

2. Materials and methods

2.1. Northern blot analysis

Two multiple tissue Northern blots (Life Technologies, Rockville, MD) containing immobilized polyA$^+$ mRNA (2 μg each) from several human tissues were probed for SPEC1 mRNA expression. Two different probes were generated, both of which were derived from the 1.2 kb SPEC1 cDNA (Pirone et al., 2000). One of the probes was derived from the 3′-end of the SPEC1 cDNA (nucleotides 574–1286), while the second probe was derived from the 5′-end of the SPEC1 cDNA (nucleotides 1–312). In each case the corresponding plasmid templates were first linearized and then used in an RNA polymerase transcription reaction with T3 RNA polymerase and [^{32}P]UTP (3000 Ci/mmol). The resulting high specific activity antisense riboprobes were each hybridized at 65°C under stringent conditions. Following hybridization, the blots were washed and exposed to X-ray film for 72 h.

2.2. Identification and characterization of SPEC1 splice forms

Several cDNA clones overlapping the 3′-end of the original 1.2 kb SPEC1 sequence (Pirone et al., 2000) were identified from a BLASTN search of the EST database. One of the clones (I.M.A.G.E. Consortium clone ID: 1627665) was obtained and sequenced on an Applied Biosystem 377 DNA sequencer by primer walking. The GenBank Accession number for the sequence of this 3.2 kb contig is AF18784.

In addition to these 3′-end overlapping clones, two cDNA clones encoding a distinct isoform, SPEC1-β, were also identified. These cDNA clones were obtained from the I.M.A.G.E. Consortium (clone IDs: 713828 and 207095) and sequenced. The sequence of one of the SPEC1-β clones (clone ID: 207095), containing a 2.2 kb cDNA insert, is available from GenBank under Accession number AF286592.

2.3. RT-PCR

RT-PCR analysis was used to determine whether SPEC1 and SPEC1-β isoforms were present in human tissues. The primers for SPEC1 amplification are: 5′-ATTTTGGCACAAACTGGGCT-3′ and 5′-CTATAAGCCCCTAGAATTGC-3′ (see Fig. 4). The primers for amplifying the SPEC1-β cDNA are: 5′-TGAGTCTCCCCACCCCCCAT-3′ and 5′-GCTCCATGGCCTATCTCGGT-3′ (see Fig. 4). To distinguish between the SPEC1-β mRNA and genomic contamination, SPEC1-β primers were designed to span intron 3 from intron 2 of SPEC1-β to exon 4 (see Fig. 4). The PCR reactions utilized a panel of cDNAs from different human tissues as template (OriGene Technologies, Inc., Rockville, MD). Thirty-five cycles of PCR were run according to the following step program: 45 s of denaturation at 95°C, 45 s of annealing at 47°C, and 1 min of extension at 72°C. As positive controls for SPEC1 and SPEC1-β amplification, plasmids containing the corresponding cDNAs were used as template. For each set of reactions a water control was also employed. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Southern blot analysis was also performed with a SPEC1-specific cDNA probe to confirm the specificity of the observed PCR products.

2.4. In vitro transcription and translation

Coding sequences for SPEC1 and SPEC1-β were cloned downstream of the T7 promoter in the pcDNA3 vector (Invitrogen, Carlsbad, CA). *In vitro* transcription and translation was performed using the coupled TNT® system (Promega, Madison, WI). In place of [^{35}S]methionine labeling, a colorimetric, non-isotopic method using biotinylated lysine charged t-RNAs was utilized (Promega). In each reaction, 2 μg each of pcDNA3, pcDNA3-SPEC1, or pcDNA3-SPEC1-β was used in a 50 μl reaction mixture containing 40 μl of rabbit reticulocyte lysate, 1 μl of

methionine, and 2 μl Transcend™ Biotin-Lysyl-tRNA. Each reaction mixture was incubated for 1 h at 30°C and biotinylated proteins were affinity-purified using Soft-Avidin-agarose as described by the manufacturer (Promega). The biotinylated proteins were then resolved on an 18% SDS-PAGE gel, transferred to nitrocellulose, and detected colorimetrically using a streptavidin-alkaline phosphatase (Promega).

2.5. Isolation and characterization of a genomic clone for the SPEC1 gene

A 0.6 kb fragment of the SPEC1 cDNA was amplified by PCR using primers specific to the SPEC1 gene sequence (forward primer, 5′-TGAGTCTCCCCACCCCCCAT-3′ and reverse primer, 5′-AGTCAGCACGGAGGAAGGGA-3′) and Vent polymerase (New England Biolabs Inc., Beverly, MA). This PCR product was gel-purified, radiolabeled, and used as a probe to screen a human BAC library (Research Genetics, Huntsville, AL) under stringent conditions. One positive clone was obtained from this screen. Using PCR analysis and DNA sequencing, this BAC clone was found to correspond to the human SPEC1 gene. Using this sequence information, the exon and intron splice junctions were determined.

2.6. Transfection and reporter assays

A 776 bp region containing the 5′-untranslated sequences of both the AF1q and SPEC1 genes and the corresponding intergenic region was amplified from the BAC clone containing SPEC1 using primers 5′-CCAGACGTTCAGC-TACAGCT-3′ and 5′-TATTCCCGATCTCTTCCAGC-3′. This sequence is available from GenBank under Accession number AY026491. The PCR product was subcloned in both orientations into the luciferase reporter vector, pXP (Nordeen, 1988). The construct corresponding to orientation for the promoter for the SPEC1 gene was designated pXP-SP while that for the AF1q gene was designated pXP-AF. For functional promoter analysis, 1 μg of either the pXP control vector or pXP promoter constructs were co-transfected along with 5 pg of the plasmid for CMV-*Renilla* luciferase. Jurkat T-cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and were transfected by electroporation (250 V, 960 μF). Cos1 cells were grown in DMEM supplemented with 10% FBS, while MCF-7 breast cancer cells were grown in IMEM supplemented with 10% FBS. Both Cos1 and MCF-7 cells were transfected using Fugene reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. NIH-3T3 fibroblasts were maintained in DMEM supplemented with 10% calf serum and were transfected using Lipofectamine Plus transfection reagent (Life Technologies, Rockville, MD). Luciferase assays were performed using the Dual-Luciferase™ Reporter Assay System (Promega) and measured on a LB9501 Berthold luminometer. Transfections were performed in triplicate and promoter activity is representative of three different experiments.

3. Results

3.1. Northern analysis reveals multiple SPEC1 mRNA transcripts

Using Northern blot analysis with a probe derived from the 3′-untranslated region of the 1.2 kb SPEC1 cDNA (Pirone et al., 2000), we detected the ubiquitous expression of three major transcripts of 1.6, 3.3, and 6.3 kb in brain, uterus, placenta, kidney, spleen and lung (Fig. 1A). In these tissues a minor transcript of 10.2 kb was also detected (Fig. 1A). To rule out non-specific hybridization, a second probe directed against the 5′-untranslated region of SPEC1 was used in a matched blot. Hybridization with this alternate probe showed an identical distribution pattern to that seen with the 3′-untranslated probe (Fig. 1B). Northern analysis using a probe derived from the coding region of SPEC1 also detected the same pattern of mRNA transcripts (data not shown). These Northern data suggest that the different sized SPEC1 mRNA transcripts are derived from alternative splicing. Furthermore, the Northern results seen with the three independent probes demonstrate that the 1.6, 3.3, and 6.3 kb mRNA transcripts all share the originally determined 1.2 kb SPEC1 cDNA sequence.

3.2. Identification of additional SPEC1 cDNAs containing an alternative 3′-end

In an effort to identify the larger SPEC1 mRNA species, we searched the EST database for cDNA clones that over-

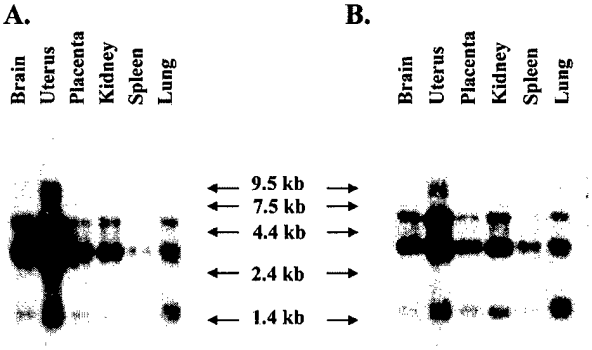

Fig. 1. Northern analysis of SPEC1 mRNA. (A) A blot containing polyA$^+$ RNA from a variety of human tissues was analyzed for SPEC1 expression using a probe derived from the 3′-untranslated region of SPEC1 (nucleotides 574–1286). Three major transcripts of 1.6, 3.3, and 6.3 kb were detected as well as a minor transcript of 10.2 kb. (B) Using a 312 bp probe derived from the 5′-untranslated region of SPEC1 (nucleotides 1–312) on a paired blot revealed a similar pattern and expression levels as that seen in (A). The molecular weight markers are indicated in kb.

lapped the previously determined 1.2 kb cDNA sequence of SPEC1. Identification and sequencing of several new overlapping SPEC1 cDNA clones revealed an additional 1.8 kb of 3′-untranslated sequence that contained no additional open reading frames. Sequence analysis of several different SPEC1 cDNA clones revealed that they utilized one of two different polyadenylation signals, one located at nucleotides 1282–1286 and the other at nucleotides 2987–2991 (Fig. 2). These results support the data from the SPEC1 Northern analysis and confirm that the 1.6 and 3.3 kb SPEC1 mRNA species are due to alternate usage of these polyadenylation sites.

3.3. SPEC1 and SPEC1-β isoforms are present in multiple human tissues

In addition to the 3′-end alternatively spliced SPEC1 clones, we also identified and sequenced an additional isoform that had an altered protein coding sequence distinct from that of SPEC1. This isoform, SPEC1-β, was initially detected as two different clones from EST database searches. The SPEC1-β translation product would encode a 38 amino acid protein, in which the first 18 amino acids would be identical to SPEC1 (Fig. 3A). SPEC1-β would also contain a unique proline rich C-terminus, ending in a CAAX box-like sequence (Fig. 3A). Specifically, the C-terminal, C-A-V-R sequence of SPEC1-β may be involved in plasma membrane targeting as it is in other proteins such as Ras (Hancock et al., 1991; Moores et al., 1991).

To examine the distribution of the SPEC1-β mRNA isoform, we performed RT-PCR with human cDNAs derived from lung, placenta, heart, kidney, liver, and colon. In these experiments, we used two different sets of primers to amplify either the SPEC1 or SPEC1-β cDNAs. Using RT-PCR, we obtained the expected 230 bp fragment corresponding to SPEC1 in all tissues examined (Fig. 3B). In the case of SPEC1-β detection, we designed primers spanning intron 3 to differentiate between SPEC1-β cDNA and potential genomic contamination (see Section 2). Using these SPEC1-β-specific primers, a 720 bp fragment corresponding to the SPEC1-β cDNA was amplified in all of the same tissues (Fig. 3B). In each case, SPEC1 and SPEC1-β products were confirmed using Southern blotting (data not shown). Based on experiments using primers common to both SPEC1 and SPEC1-β, we consistently observed a much lower level of SPEC1-β expression as

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   1  CAGAGCTAGCCCGGGAAGCCCACACTGGCGGCCACGGAGCAGAGTCCCTCACCCCCACCAGCTGTAGCTGAACGTCTGGATGGTGGAGAA
  91  GAGCAGGGTTCCGAGTCTGAGGAAGACATAACCTTGTGCCTGCCTGCCCACCTCTCTCTCTGGTCCTGTTCATCTCTCAGGCTCTGAGAC
 181  ACTGACCTTCACTGCTCAGTTAAAGGTTCCAGGGATTCCACTTTGTCTGGACCCATCCAGCTGAGTGAACCCAGGGTGGTGGTGTATCTG
 271  GGGAGAGTGAGGAGTGGGTTGTCCAAACACCAGGGAAAGAGCCCTTTGGGGCCTCAGACAGAGGAGTGAAGCTGGAACCATCAGGGAACA
                                                                                          M   1
 361  TGAGTGAATTTTGGCACAAACTGGGCTGCTGTGTGGTAGAGAAACCCCAGCCGAAGAAGAAGAGAAGACGGATTGACCGGACCATGATTG
        S   E   F   W   H   K   L   G   C   C   V   V   E   K   P   Q   P   K   K   K   R   R   R   I   D   R   T   M   I   G  31
 451  GGGAACCAATGAATTTTGTTCACCTGACTCACATTGGCTCAGGGGAGATGGGGGCCGGAGATGGACTTGCCATGACAGGTGCAGTTCAGG
        E   P   M   N   F   V   H   L   T   H   I   G   S   G   E   M   G   A   G   D   G   L   A   M   T   G   A   V   Q   E  61
 541  AGCAGATGAGATCCAAGGGAAACCGAGATAGGCCATGGAGCAATTCTAGGGGCTTATAGCTCCAATAATGGAATGGTTCTGCCATCTTGA
        Q   M   R   S   K   G   N   R   D   R   P   W   S   N   S   R   G   L   *                  79
 631  AACCCCCATTCTGTTTCCAGCCCAGAAGAAATGCTGCCCCTACCAGATCCCTCCTTGAACCAGTGATCTAAGGACCCCTCTTTTCCCTAT
 721  CTGCCTAACAGTGCCTCACAAGGCTTGGGGGCTGGACTCCCTCTACTCCCTCTGGCCATAGCCCCTCCTGGAGATGGGGTCAAGGCAGCA
 811  GGACTGATCAAGTGACTACTGGTTAGCCAGAGGGAGCGCTGAAGCCTAGGAAACCCTCAGGTCTGAGATAGGAGTTCTCTAGGAACCTGG
 901  AATGAGTTCCTGTCTCCTGAATGATGGTCTGGGTGCCACCTGTTTTTAAACTCTTAAACCTGGAACTCCTTAAATGGGGTAGGTGGGTGA
 991  GATTATCAAAGCTGAAGCTGGCTTTGCTGAGAAGCTCCCTACCTCCCTGCCCTTCTCCTCCTTCCTGGCTGGGAATGAACTAAAGCAGAT
1081  GTCTAAGCAGGGGCTGGGGGGGGTGCCTACTCCCTTTTCCACTCTATCTTTAGATTTCAAACCTTAGGCTTACAGCCCTCAATATCTCTCT
1171  GCTAACACCAGTGTCTCTTTCTAGTTAGGCCTCTAATCTTCTGTTTCTGTTTACCAGCTTCCCAGCAACTTTCCTTTTTTAAAATATTAA
1261  AAATTTAATTCAGGTTCTCTTAATTATCCTCATCCTGCTGTTCCTCCGCCCTTAACTCTATCCCTATTAGGAACCTTGTTTCCCACCGAA
1351  TAAGAGTTAAGGTAAGAGGTAGCTCTTCACCTGATGACATTCCATTTAAGTTTTGGGGCATCTTTCCTGCCCTCTGCCAACCTCCCTGCT
1441  GGCCCAGGTGAGAGGAGGAAGAGGGGTCTGGAAAGAAACCCAGAATGCGGCAGAACTAGGAGTAAGTCCATCTCAAGGTGGACTTCTGAT
1531  CATTGAGGATGGTGAGATAAATAGGTTGAAAACAATCATCTGTGAGTTTAGGAGTCTTAAGATCCTCACAGCACGGGAGCAGGGAGAGGC
1621  TCTAGAACAAGGTTTCACAACCGAGATGCCTTTGACATTTTGGGCTGAAGTGTTCTTTGTAAGGAAAACCTGTTTCCTATTTTGTGCATT
1711  ACAGGATATTTAGCAGCACGCCTGGCCCACTCTAGTTATGACAACCAAAAATGTCTCTATACATTGCCAGATTTCTCCTAGGGGGCAAAA
1801  TCGCCTCCATTTGAGACTACAACTCTGAGTTCCGTAGGGGGCAAAATCGCCTCCATTTGAGAACCACAACTCTAAAGTCAAGATATTTTT
1891  CTCTGAAGTGATTTCTGCTTGGCACGCTACCCTCATCATCTTACTCATCTTAAGCCCGAGCATGGATCAACTAGGAGCCCATATAAATGA
1981  GGCTCAGTAGGGCTGACATAATCTCCAAACAATTCTTTAATTCAGTGTATTGCTTGGTGTTTGCATGTTGCTATGATTGGAACAAGGTTC
2071  CCCCTCCTCCATTCTTGCCCTCTGGAGAAGTGGTTCCTGCAGTGTAGAGATATGAACAGGGTATGGTAGGATTGGGGAAAGGGGAGAGAG
2161  AAGCAGTAGATACACTCCTTATCCTCCCCAAATTTTAAGCTCTATTTTTGTGCCCTAGTCCTAGACACACATTAGACTCAGGGAGTTTTG
2251  TCTGAAGACCAGGTCCCACTGCCCCCCTGGCTGAAGAGTCTGCTTTAAATGGGAAAACAACGTAGGAGCAGGGGTTTTTAGGCACTTTCAG
2341  TTTTCTCAGGTGTTCTTCGTTCTGGCCCTTCCAGGGTAATTAGGAAGGCAGAACAAGACAGATGAGCTCCTGCCTGCTCTGAGACAAGAA
2431  GGGTGGGGTCTCATTAGCTTTGCAACAGGAAACATCCTGTTTTATTATGGTAGTGGGGTCAGGAATGTAGGAACTGGTATCCATTCTGCC
2521  AATTCCCACCCATTCAGTTTGCTTATCCCTACAGAACAGTGACTGAGGTTCCTTTTTTTTTTTTTTTTTTTTTTTTTTTCAAATTTCCAT
2611  GTATTTTCTGCCATTTTTCAGGGTCTAAGATTGGTCATACATTCCCAATTTACTCTCAGTTCCAGTCAAGCTGGTTGCTCTGAAAGTAAC
2701  CCAGCTTGTTGCTCTAAAATACCTCAGTAGCCTGAGTGTTATACTAGAGATCTAAAGGGTTAACAGGATAGGGTGGAAAGGTTAGAGACT
2791  CCTAGAAATCTCTGGTCACCGTGATCTTCGGCCTCATTCTAATACCTGTTCTTTGGACAGTCTTTTTCCTTTGGTGCTCTCTTGCCTTTA
2881  GCTACCTTCTCTAATATGTATGCTACCATCACTAATAAAGTGATGGGAATGGGTTTGAGAGTCGTAATTTATATTAAAAAGTTGTTGGAC
2971  TTTTAAATACATTTTTTCAAATAAAAAATTAAGCAAAAT
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Fig. 2. Nucleotide and amino acid sequences of the human SPEC1 cDNA. Nucleotides are numbered on the left; amino acid residues are numbered on the right. The sequence shown is a composite of several overlapping clones. Analysis of various cDNA clones revealed that they encoded two different mRNA species, which arose from different 3′-polyadenylation signals. The two different polyadenylation signals are underlined.

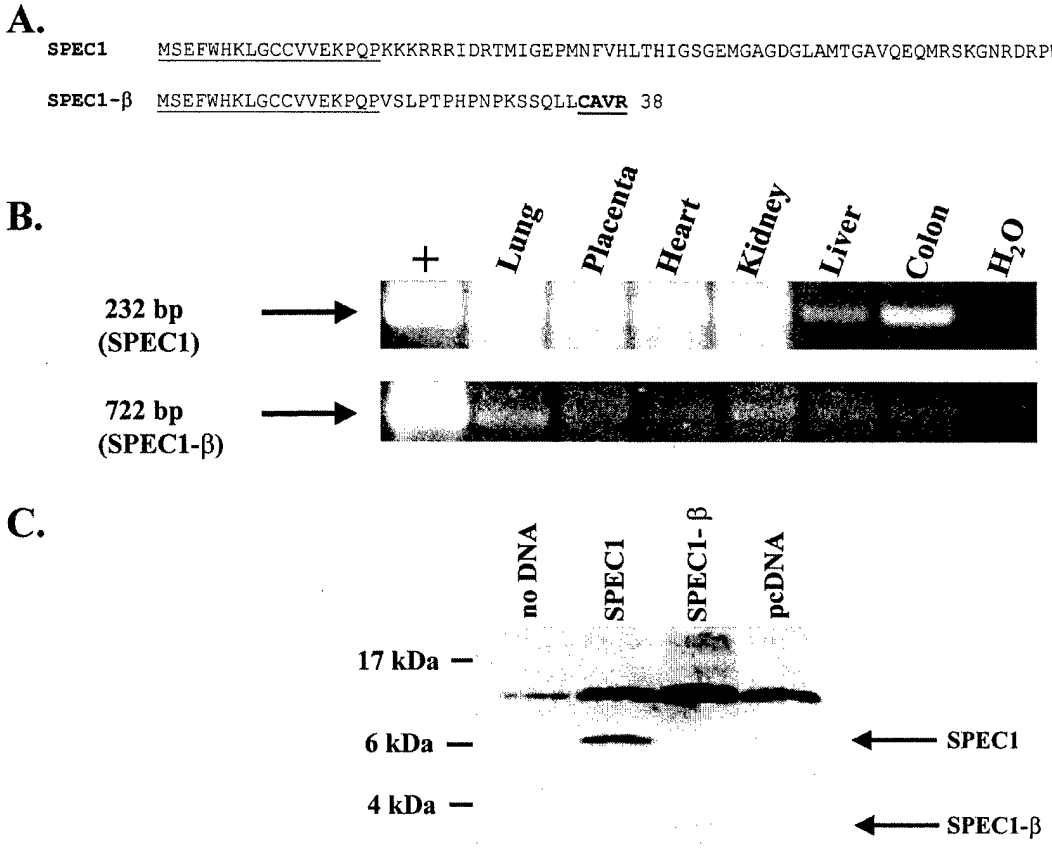

Fig. 3. Identification and expression of SPEC1-β. (A) Amino acid alignment of SPEC1 and SPEC1-β proteins. The numbers indicate the size of the proteins in amino acid residues. The N-terminal 18 amino acids which are common to SPEC1 and SPEC1-β are underlined. The potential CAAX motif of SPEC1-β is in bold font and underlined. (B) Expression analysis of SPEC1 and SPEC1-β mRNA by RT-PCR in a variety of human tissues. PCR-amplified products were resolved on a 1% agarose gel and stained with ethidium bromide. The + denotes the appropriate positive controls generated from SPEC1 and SPEC1-β plasmid clones. The sizes of the PCR products are indicated to the left of the gel. (C) *In vitro* transcription and translation of SPEC1 and SPEC1-β cDNAs. The coding regions of both SPEC1 and SPEC1-β were cloned into the pcDNA3 vector and used for *in vitro* transcription and translation. In these reactions biotinylated lysine residues were incorporated into the protein products. Translated products were separated on an 18% SDS-PAGE gel, transferred to nitrocellulose, probed with streptavidin-alkaline phosphatase conjugate and detected colorimetrically. The molecular weights are indicated to the left of the blot. The arrows to the right of the blot indicate the positions of the SPEC1 and SPEC1-β protein products.

compared to SPEC1 (data not shown). These results confirm the presence of SPEC1-β, but suggest that it is present only as a minor transcript.

Because of the unusually small size of the SPEC1-β mRNA isoform, we used *in vitro* transcription and translation to examine whether the SPEC1-β cDNA could generate a protein product. In these experiments, plasmid constructs were generated containing SPEC1 or SPEC1-β cDNAs downstream of the T7 promoter. In this *in vitro* transcription and translation system, the incorporation of biotinylated lysine residues was used to monitor protein production. Analysis of the translation products on an 18% SDS-PAGE gel revealed that SPEC1 and SPEC1-β generated 8 and 4 kDa protein species, respectively (Fig. 3C). In contrast, no protein product was observed with the sample containing no DNA or with the empty vector control (Fig. 3C). These results support the possibility that the SPEC-β protein is translated *in vivo*.

3.4. Characterization of the SPEC1 exon–intron structure

To further understand the organization of the SPEC1 gene and the origin of the mRNA splice variants, a single BAC clone was isolated from a genomic library with a SPEC1-specific probe. While this work was in progress, the working draft sequences of a bacterial artificial chromosome clone (RP11-316M1) containing the SPEC1 gene became available through the Human Genome Project and we have used these data in conjunction with our sequence data to determine the exon–intron organization of the SPEC1 gene. Characterization of the SPEC1 BAC clone by DNA sequencing and PCR analysis revealed that both the entire 1.6 and 3.3 kb SPEC1 cDNAs were encoded by six exons (Fig. 4). Of these six exons, only exons 2, 3 and 4 encoded the 79 amino acid residues of the SPEC1 protein. Exon 2, consisting of 320 bp, contained the start methionine and the first 18 amino acids of the SPEC1 coding sequence (Fig. 4). Exon 3

was 111 bp and encoded the entire CRIB domain responsible for Cdc42 binding, while exon 4 encoded the C-terminal 24 amino acids of SPEC1 and was 93 bp long (Fig. 4). The difference in the 3′-ends of the 1.6 and 3.3 kb species was due to alternative splicing in exon 6, whereby an additional 1.8 kb of 3′-untranslated sequence generates the 3.3 kb cDNA. We also compared the SPEC1-β cDNA sequence with the genomic sequence of SPEC1 to formally prove that the SPEC1-β cDNA was derived from the SPEC1 gene. Comparison of these sequences revealed that the 0.5 kb intron located between coding exons 2 and 3 was retained in the SPEC1-β cDNA, although other introns in the SPEC1 gene were properly spliced (Fig. 4). Finally, using fluorescent *in situ* hybridization the SPEC1 gene was localized to human chromosome 1q21.1-1q21.3, consistent with the sequence and mapping data of the Human Genome Project (data not shown).

3.5. SPEC1 and AF1q genes are in a head-to-head arrangement

In the process of characterizing the genomic sequence flanking exon 1 of SPEC1, we identified that the AF1q gene is in close proximity to the 5′-end of the SPEC1 gene. AF1q encodes a small protein of 90 amino acids of unknown function that was previously discovered as a translocation partner with the MLL gene in patients with acute myelomonocytic leukemia (Tse et al., 1995; Busson-Le Coniat et al., 1999; So et al., 2000). The AF1q message of 1.8 kb is restricted to hematopoietic cells, unlike SPEC1 (Tse et al., 1995). Alignment of the genomic sequences of these two genes revealed that SPEC1 and AF1q are in a head-to-head orientation separated by a distance of 631 bp (Fig. 5). Further sequence analysis of this intergenic region revealed that this sequence did not contain a TATA or CAAT box. However, putative binding sites for several transcription factors were identified, including a GC box as well as putative CdxA, GATA-3, and CP2 binding sites (Fig. 5).

3.6. The intergenic region between SPEC1 and AF1q genes has SPEC1 promoter activity

To test whether this region was transcriptionally active for both genes, a 776 bp segment containing the putative promoter region as well as some 5′-untranslated sequence from both SPEC1 and AF1q genes was subcloned in both directions into a promoterless luciferase reporter construct, pXP. The construct corresponding to the promoter for SPEC1 was designated pXP-SP while that of AF1q was designated pXP-AF. Transfection analysis of the SPEC1 promoter construct revealed that it was highly active in Cos1 and MCF-7 cells, weakly active in NIH-3T3 fibroblasts and inactive in Jurkat T-cells. Furthermore, a relatively similar level of activity of the CMV-*Renilla* luciferase construct was observed in each cell type. The AF1q promoter construct showed only background levels of luciferase activity similar to the pXP vector control construct in all cell lines tested (Fig. 6). Interestingly, the SPEC1 promoter appears to have some level of tissue-specific regulation. In particular, the SPEC1 promoter was highly active in Cos1 cells and MCF-7 breast cancer cells, but was less active in NIH-3T3 fibroblasts and inactive in Jurkat T-cells (Fig. 6). These results suggest the possibility that the SPEC1 promoter exhibits epithelial tissue specificity. Using Northern analysis, we have confirmed high

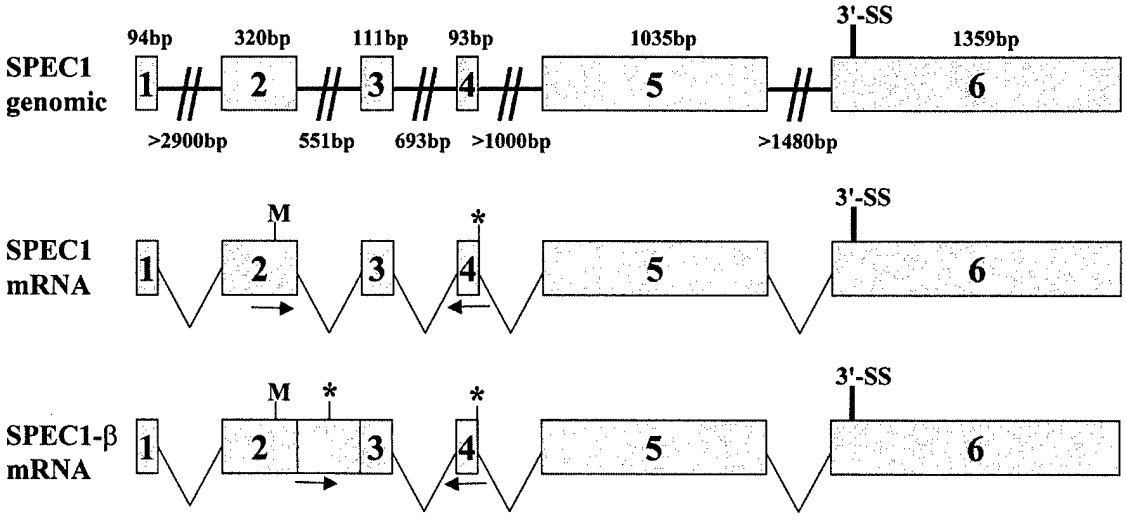

Fig. 4. Genomic structure of the SPEC1 gene. The boxes represent the six exons of the SPEC1 gene, while intron sequences are indicated by a thin line. The site of the 3′-end alternative splicing in exon 6 is indicated by a bar labeled for the 3′-splice site (3′-SS). Lengths of exons and introns are indicated in bp. In addition to the genomic organization corresponding to the SPEC1 cDNA, the origin of the SPEC1-β mRNA product is also shown involving the retention of intron 2 as denoted by the hatched box. Met indicates the start of translation and the asterisk (*) indicates the stop codon. The arrows below the schematics of SPEC1 and SPEC1-β mRNAs correspond to the primers used for RT-PCR.

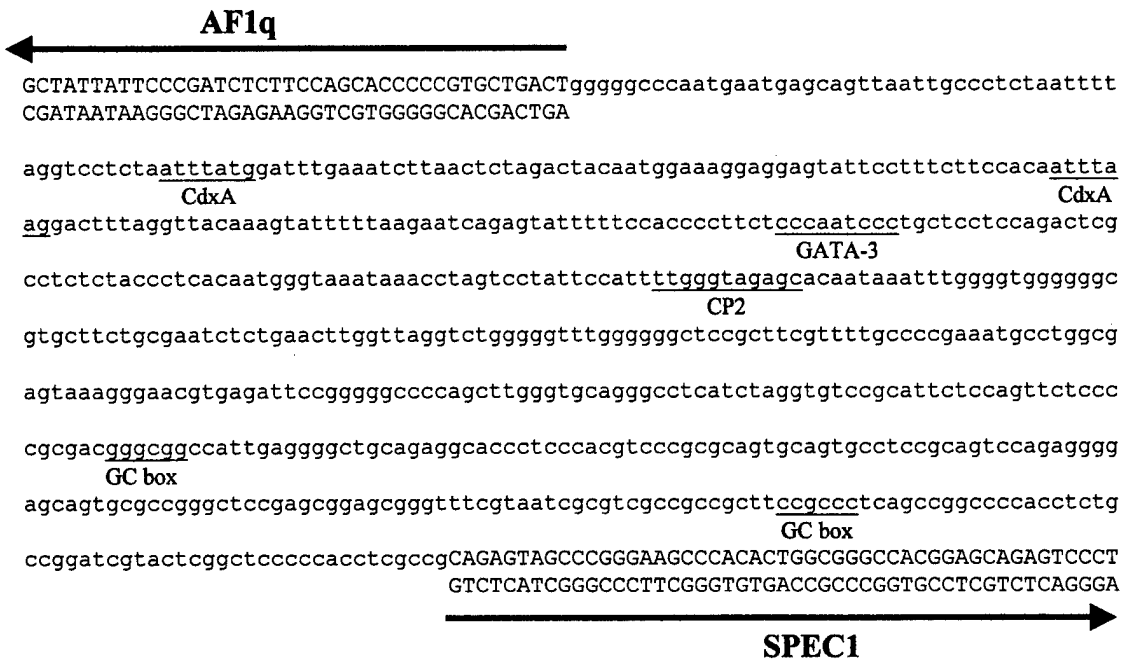

Fig. 5. DNA sequence of the intergenic region between the SPEC1 and AF1q genes. The nucleotide sequence is numbered to the right. The capital letters indicate the 5′-untranslated region of the SPEC1 and AF1q genes while the small letters represent the intergenic sequence located between both genes. The intergenic region begins at nucleotide 41 and ends at nucleotide 671. The arrows indicate the direction of transcription for each gene. The putative binding sites for several transcription factors are underlined.

levels of SPEC1 mRNA expression in a variety of breast cancer cells (data not shown).

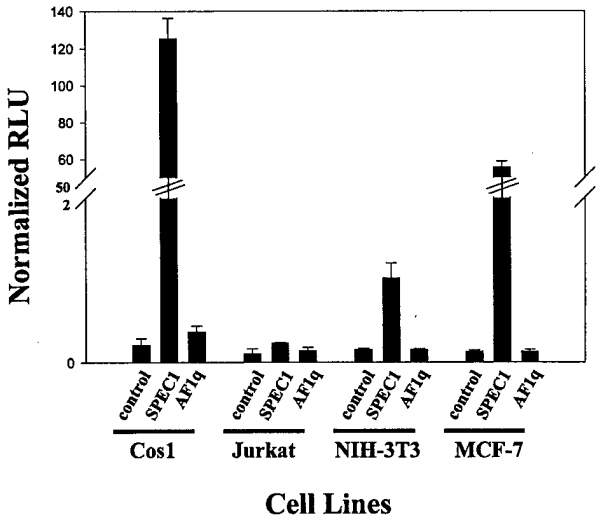

Fig. 6. SPEC1 promoter activity. Promoter constructs for both SPEC1 and AF1q were constructed in the promoterless luciferase reporter vector pXP. The control vector (pXP), the promoter for SPEC1 (pXP-SP), or the promoter for AF1q (pXP-AF) were co-transfected into Cos1, NIH-3T3, Jurkat T, and MCF-7 cells with a construct for CMV-*Renilla* luciferase. Luciferase assays were performed 24 h post-transfection. Promoter activity is reported as normalized relative light units (RLU) as compared to *Renilla* luciferase activity to normalize for transfection efficiency. Luciferase assays were performed in triplicate and are representative of three different experiments. Error bars indicate standard deviation.

4. Discussion

The SPEC family of proteins, consisting of SPEC1 and SPEC2, represents the smallest known Cdc42-binding proteins (Pirone et al., 2000). Here we have delineated the genomic structure of the SPEC1 gene, and have determined that it contains six exons spanning over 10 kb. Of these exons, only exons 2, 3, and 4 encode the 79 amino acids of the SPEC1 protein. Interestingly, the structure of the human SPEC1 gene is very similar to that of the human SPEC2 gene located at 5q31 (Pirone et al., 2000), whereby three similar sized coding exons encode the full-length proteins. One marked difference in the genomic structure between SPEC1 and SPEC2 is the presence of a 26 kb intron between coding exons 1 and 2 of SPEC2 (Pirone et al., 2000), while only a 551 bp intron region is found between the corresponding exons of SPEC1. Based on these similarities in the genomic structure of the coding regions of the SPEC genes, it is likely that both SPEC genes evolved from one another.

In the course of these studies, we have identified heterogeneity in the 3′-untranslated sequences of the SPEC1 mRNA producing transcripts of 1.6, 3.3, and 6.3 kb. Although the functional consequences of these different 3′-end mRNAs are not known, they may show altered stability or cellular localization. In addition to the alternative splicing in the 3′-end of the SPEC1 mRNA, we identified by EST database searches an alternatively spliced isoform from within the SPEC1 coding region. This isoform, SPEC1-β, was detected by RT-PCR as a minor transcript

in a variety of human tissues. Interestingly, the mechanism by which the SPEC1-β mRNA transcript is produced involves intron retention. Intron retention occurs in other genes and frequently occurs in cancer. For instance, there is an aberrant inclusion of intron 9 in CD44 transcripts in tumor tissues, including bladder cancer, breast cancer, ovarian cancer, and gastrointestinal tumors (Matsumura et al., 1995; Yoshida et al., 1995; Bolodeoku et al., 1996). Although SPEC1-β is present at low levels in a variety of normal tissues, it is possible that this isoform may occur more frequently in tumor tissues. Experiments using *in vitro* translation confirmed that the SPEC1-β cDNA generates the expected 3.8 kDa protein product. Since the SPEC1-β protein lacks a CRIB domain involved in Cdc42 binding and contains a potential membrane targeting sequence, it may function as a signaling molecule independent of Cdc42 control. Taken together, these results suggest the possibility that the SPEC1-β protein may exist *in vivo* and may have its own unique biological activities.

Genomic analysis revealed that the AF1q gene is in a head-to-head orientation with SPEC1. AF1q is particularly interesting, because it is involved in translocations with the MLL gene and encodes a similarly small protein of 90 amino acids (Tse et al., 1995). However, unlike the ubiquitous mRNA expression of SPEC1, expression of the AF1q mRNA is restricted to hematopoietic cells (Tse et al., 1995). Although the SPEC1 gene is in close proximity to AF1q, the site of translocation with the MLL gene occurs within the first intron of AF1q (Tse et al., 1995). Analysis of the intergenic sequence between the two genes revealed promoter activity only for the SPEC1 gene. The lack of promoter activity for the AF1q gene suggests the likely possibility that additional regulatory elements are required. Along these lines, the first intron of AF1q may contain enhancer elements needed for transcriptional activity. This possibility is also supported by the fact that translocations in other genes frequently occur at sites where the chromatin is open correlating with transcriptionally active regions (Nikiforova et al., 2000; Savage, 2000). Future experiments are aimed at examining whether the first intron of AF1q contains a hematopoietic-specific enhancer.

Acknowledgements

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Evolutionary expansion of CRIB-containing Cdc42 effector proteins

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Cdc42, a small GTPase, regulates actin polymerization and other signaling pathways through interaction with many different downstream effector proteins. Most of these effector proteins contain a Cdc42-binding domain, called a CRIB domain. Here, we describe the evolutionary analysis of these CRIB-containing proteins in yeast, worms, flies and humans. The number of CRIB-containing effector proteins increases from yeast to humans, involving both an increase within families and the emergence of new families. These evolutionary changes correlate with the development of the more complex signaling pathways present in higher organisms.

Understanding the molecular mechanisms by which lower organisms such as yeast, worms and flies regulate intracellular signaling cascades could provide insights into the signaling mechanisms used in humans. However, several of the signaling components and protein domains present in humans are completely missing from some of the best-studied model organisms. For example, phosphotyrosine-SH2 domain signaling has a major role in invertebrates and vertebrates, but is absent in yeast[1]. By contrast, some signaling pathways, such as those involving the Cdc42 GTPase, are conserved in all eukaryotes. Cdc42 functions to link cell-surface receptors to intracellular signaling pathways regulating cell shape, actin polymerization, protein trafficking and kinase signaling[2,3].

Similarly to other GTPases, Cdc42 interacts with numerous different downstream effector proteins to cause the various responses[3,4]. Molecular cloning of Cdc42 effector proteins from a variety of different organisms reveals that many contain a common Cdc42-binding domain, the CRIB domain[5] (also known as GBD and PBD). These CRIB-containing effector proteins are structurally and functionally diverse and include serine/threonine kinases, tyrosine kinases, actin-binding proteins and adapter molecules[3,4]. In addition to Cdc42, several related GTPases, such as Rac, TC10 and TCL, can also interact with some of these same CRIB-containing proteins[5–7]. Here we summarize evidence indicating that CRIB-containing proteins offer interesting insights into the evolution of signaling networks.

Although CRIB domains are present in many different proteins from a wide variety of species, existing protein domain databases, such as SMART (Ref. 8) or Pfam (Ref. 9), are either incomplete or do not include the CRIB domain. Here, we used BLASTP and TBLASTN (Ref. 10) searches of the nonredundant protein, nucleotide and genomic sequence databases to identify most or all of the CRIB-encoding genes in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. In these searches we used only the core CRIB domain,

I(S/G)XPX$_{(2-4)}$FXHXXHVG, although it is known that additional amino acids surrounding the core sequence participate in GTPase binding. A table listing the sequences of all the identified CRIB domains and corresponding accession numbers is available at http://bc.georgetown.edu/pb/crib.html.

As previously described[11], a search of the *S. cerevisiae* genome revealed five CRIB-containing Cdc42 effector proteins (Fig. 1), three of which, Ste20, Skm1 and Cla4, belong to the p21-associated kinase (PAK) family[3]. The two others, Gic1 and Gic2, are structurally related, non-kinase effector proteins[11] that are not apparently homologous to any *D. melanogaster* or mammalian proteins, although homologs of these molecules are found in plants (data not shown). These comparisons suggest that Gics might have more specialized roles in cytoskeletal changes, such as those leading to cell-wall assembly.

In *C. elegans*, eight genes encoding CRIB-containing proteins were identified (Fig. 1). Of these, four encode presumed kinases and four encode presumed non-kinases (Fig. 1). The PAK-like kinases are encoded by two genes, and single genes encode ACK (Ref. 12) and MRCK (Ref. 13) homologs. The four non-kinase effector proteins share no common structural features except for the CRIB domain. Two of these non-kinase proteins have homologs in the WASP (Refs 14,15) and PAR-6 (Ref. 16) families, which in humans function in actin reorganization and cell polarity. The two other non-kinase CRIB-containing proteins are completely novel and do not have any known structural homologs in yeast, flies or humans.

Nine genes encoding seven families of CRIB-containing proteins were found in *D. melanogaster* (Fig. 1). Six of these nine genes contain regions homologous to four known families of protein kinases including PAK-like kinases (three genes), and single genes for each of the dACK (Refs 12,17), dMRCK/GEK (Refs 13,18), and dMLK (Ref. 19) family homologs. Like *C. elegans*, *D. melanogaster* also contains WASP (Refs 14,15) and PAR-6 (Ref. 16) homologs. In addition, a single SPEC (Ref. 20) homolog is found in *D. melanogaster*, but not in *C. elegans* (Fig. 1).

In humans, 25 genes encoding nine distinct CRIB-containing protein families were identified (Fig. 1). This large and diverse group includes four families of kinases (12 members) and five families of non-kinase effector proteins (13 members). Although no new CRIB-containing kinase families were found in humans as compared with *D. melanogaster*, there was a doubling in the numbers of proteins in most of the families, including six genes for PAK kinases, three genes for MRCKs and two genes for MLKs. There was also an increase in the size of each family of non-kinase effector proteins. Although there is a single non-kinase effector in each *Drosophila* family, in humans there are three PAR-6 genes, two WASP genes and two SPEC genes.

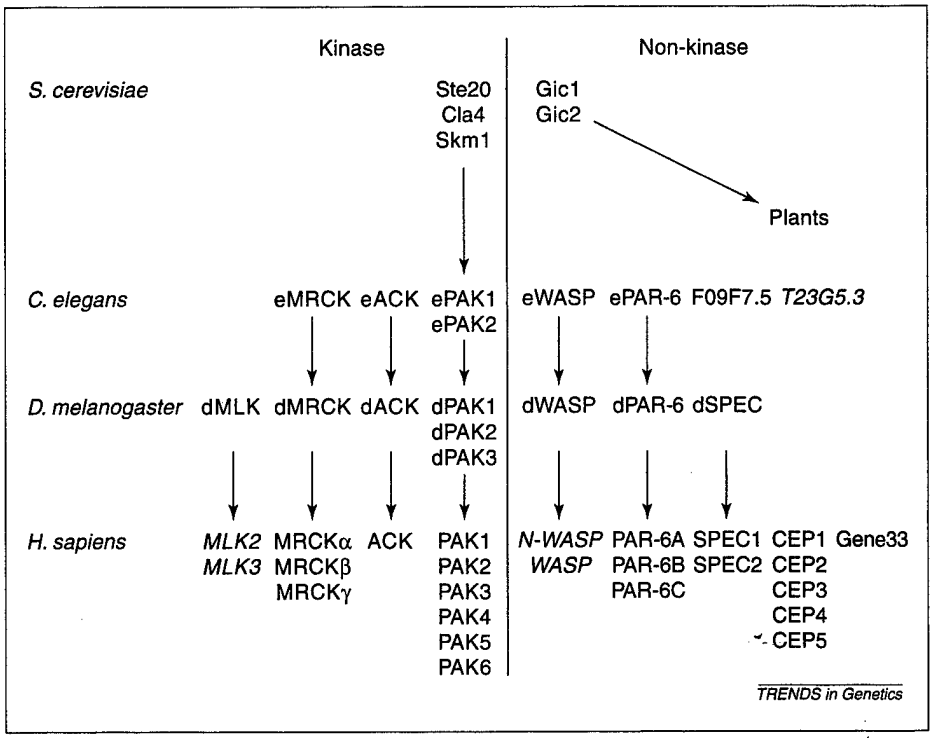

Fig. 1. The evolution of CRIB-containing proteins in eukaryotes. Kinase effector proteins are shown to the left of the dotted line, and non-kinase effector proteins are shown on the right. Arrows imply orthology between the different *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens* proteins. Ste20, Skm1 and Cla4 from *S. cerevisiae* have a similar kinase domain to that of the p21-associated kinases (PAKs). No invertebrate or vertebrate homologs are known for Gic1 and Gic2. Italicized protein names represent genes with split exons encoding the CRIB domain.

In addition, genes encoding two unique families of human non-kinase Cdc42 effector proteins were identified including a single gene for Gene33 (Ref. 21) and five distinct members of the CEP/Borg (Refs 22,23) family (Fig. 1). The larger repertoire of CRIB-containing proteins in humans indicates that Cdc42 signaling in humans is likely to be more complex than in *Drosophila*. In particular, it seems possible that many of the 25 CRIB-containing human proteins might co-exist in the same cell and might have similar affinities for Cdc42. Understanding how different CRIB-containing effector proteins compete for a limited supply of Cdc42 and comparing these mechanisms to the simpler situation in *D. melanogaster* and *C. elegans* is likely to reveal important insights into both the operation and evolution of complex signaling pathways.

Phylogenetic analysis was used to investigate the evolutionary relationship among the human CRIB domains. The most striking feature is that for most of these genes, the CRIB domains are localized to single exons (Fig. 1). Because all these exons encode additional amino acids at both the N- and C-terminal ends of the CRIB domains, we analyzed the amino acid sequences of the CRIB domains along with a few adjacent amino acids using an unrooted phylogenetic analysis with the neighbor-joining method. The three human PAR proteins were excluded from this analysis because they contained only half a CRIB domain. The resulting phylogenetic tree groups the remaining 22 human CRIB-containing proteins into six distinct evolutionary groups (Fig. 2). These CRIB domains can, in general, be assigned to evolutionary groups that reflect the overall homology of the larger protein in which they reside. Thus, the CRIB domains in the human

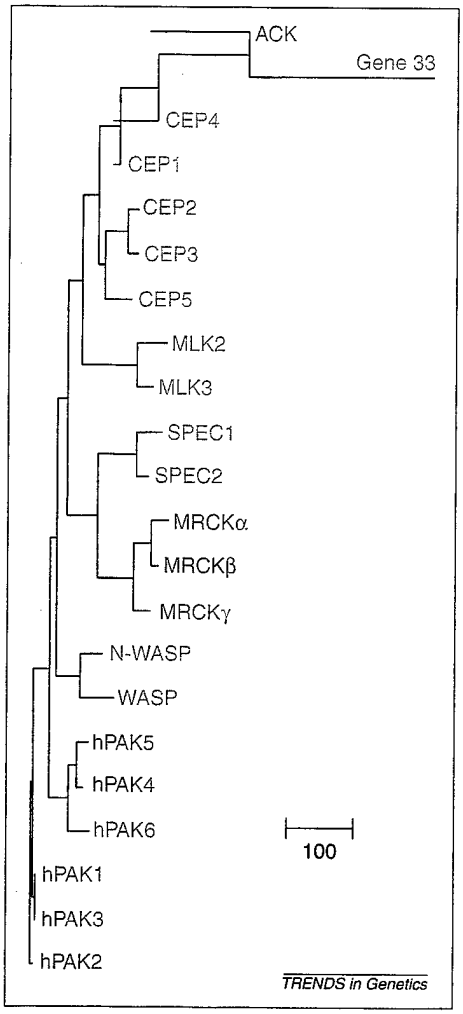

Fig. 2. Phylogram of human CRIB-containing proteins. The amino acid sequences of human CRIB-containing proteins were aligned using the Pileup algorithm of the GCG program (http://www.gcg.com). The dendogram represents the degree of homology between each sequence and was created using the GrowTree program. Kinases are shown in red, and non-kinases are shown in blue. The scale bar shows 100 substitutions per 100 residues.

PAKs segregate into two groups; one for PAK1–3 and one for PAK4–6 (Fig. 2). Similarly, separate groups are formed by the CRIB domains in the two WASP proteins, the CRIB domains in the three MRCKs and the CRIB domains in the two SPEC genes. The CRIB domains in the two MLKs and in the five CEPs also segregated into separate groups.

The tree also suggests that the CRIB domains in the non-kinase effector protein Gene 33 and the ACK tyrosine kinase are most closely related to the CRIB domain in CEP4 (Fig. 2). We note that the kinase and non-kinase effector proteins do not cluster into two larger groups. For example, the CRIB domains of the MLK kinases are more closely related to the CRIB domains of the non-kinase CEPs than to the CRIB domains of any of the other kinase groups.

Overall these analyses suggest the possibility that the CRIB domains within these six different groups might have evolved from a common ancestor that is most similar to the present day PAKs. One possibility is that gene duplication followed by exon shuffling was involved in the evolution of CRIB-containing proteins. However, if true this tree also suggests that a single exon-shuffling event between kinase and non-kinase genes is not sufficient to account for this clustering together of kinase and non-kinase effectors. Other kinds of evolutionary events must also have occurred because the exons encoding the various CRIB domains have a variable number of nonhomologous additional amino acids at both ends of the CRIB domains in each of the families examined (data not shown). These observations suggest that these additional amino acids are nonfunctional or that they might impose subtly different biochemical properties on the CRIB domains.

These analyses reveal an increasing diversity of downstream CRIB-containing Cdc42 effector proteins from yeast to humans. The increased intricacy and number of CRIB-domain Cdc42 effector proteins is consistent with other comparative genomic studies showing that both the number and complexity of multidomain structures increases with an organism's complexity[24]. The enlarged repertoire of these effector molecules in humans and presumably other mammals probably contributes in several ways to the increased complexity of higher organisms: it might allow additional cell types and cell shapes, as well as more complex and subtle types of cell migration and responses to extracellular stimuli.

At least two major expansions of CRIB-containing proteins occurred in the evolution of the animal kingdom, resulting in an increase in the number of distinct families and an increase in complexity of each family. The earliest of these expansions probably occurred during the transition to multicellular organisms, resulting in the appearance of six new families of CRIB-containing signaling-molecule proteins both in *C. elegans* and in *D. melanogaster*, but not in yeast. The acquisition of these additional families of Cdc42 effector molecules was probably required for the evolution of more complex signaling pathways and for using the additional cell-surface receptors presumed necessary for the evolution of multicellular organisms and functional differentiation.

The second expansion, which might have occurred during the transition to vertebrates, resulted in the enlargement of existing families and the creation of a few new families. In particular, the two new families that occur in humans but not in *D. melanogaster* are non-kinase effectors: a single gene in the Gene33 (Ref. 21) family and the family of five CEP/Borg (Refs 22,23) proteins (Fig. 1). It is tempting to speculate that these two families might have been essential for the evolution of vertebrates. In so far as this speculation is valid, it might contribute to the resolution of a currently controversial topic: whether 'changes in the *cis*-regulatory system of genes more often underlie the evolution of morphological diversity than do changes in gene number or protein function.'[25] In any case, CRIB-containing effector proteins represent an excellent system for studying the interplay between the evolution of genes, proteins and signaling pathways and the evolution of the organisms that encode these effector proteins. Indeed, success in identifying the phenotypes induced by the actions of these proteins (e.g. filopodia formation and kinase signaling) will undoubtedly enable us to make more accurate and detailed speculations about the evolutionary process itself.

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Selective constraint in intergenic regions of human and mouse genomes

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We aligned and analyzed 100 pairs of complete, orthologous intergenic regions from the human and mouse genomes (average length ~12 000 nucleotides). The alignments alternate between highly similar segments and dissimilar segments, indicating a wide variation of selective constraint. The average number of selectively constrained nucleotides within a mammalian intergenic region is at least 2000. This is threefold higher than within a nematode intergenic region and at least twofold higher than the number of selectively constrained nucleotides coding for an average protein. Because mammals possess only two- to threefold more proteins than *Caenorhabditis elegans*, the higher complexity of mammals might be primarily because of the functioning of intergenic DNA.

In bacteria, only ~15% of the genome does not code for proteins. However, this figure increases to 30%, 70% and 95% in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Homo sapiens*, respectively. Thus, the fraction of noncoding DNA, and perhaps its functional importance, increases with the complexity of an organism. Because the function of noncoding DNA remains poorly understood, interspecies comparison is often the only way to demonstrate that a segment of such DNA is functionally important – this DNA will have evolved slowly because of negative selection[1–8].

We used a comparative approach to study selective constraint on noncoding DNA in pairs of ORTHOLOGOUS (see Glossary) human and mouse COMPLETE INTERGENIC REGIONS flanked by successive orthologous protein-coding genes. Previously, this approach revealed many functionally important segments in both transcribed (5′- and 3′-untranslated regions of mRNAs) and untranscribed noncoding sequences[1–7]. However, estimating the fraction of selectively constrained nucleotides within the total intergenic DNA requires analyzing many complete intergenic regions and has so far been performed only for nematodes[8,9].

We analyzed the pattern of similarity within 100 pairs of intergenic regions located in 37 different places on 12 human and 12 mouse chromosomes. All the sequences were from GenBank. We confirmed the SYNTENY of the compared long sequences with BLAST (Ref. 10). GenBank accession numbers of the analyzed human and mouse sequences, together with the locations of orthologous intergenic regions in them and descriptions of flanking genes, are available at ftp://ncbi.nlm.nih.gov/pub/spacer/. We identified flanking genes by annotations, available in 80% of cases, or by aligning

Glossary

Complete intergenic region: the whole DNA sequence between protein-coding regions of two successive genes.
Hit: a highly similar segment of alignment where similarity exceeds 50%. These regions are separated by 'interhits'.
Orthologous: Sequences from different species that can be traced back to a common ancestor.
Selective constraint (*C*): Within a segment of sequence, this is the fraction of nucleotides that remains invariant due to stabilizing selection[14], calculated as described in Ref. 8.
Similarity (*s*): This is the number of matches in a pair of aligned sequences divided by the length of the shorter sequence.
Synteny: Conservation of the order of genes between different species.

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Addendum to the Third Annual Summary for DOD Grant DAMD17-99-1-9201 (Originally Submitted in July 2002)

Specific Aim 1: We will test the hypothesis that SPEC1 and SPEC1-β proteins affect actin organization, cell shape, and cell movement in breast cancer cells.

Summary of Specific Aim 1: Specific Aim 1 was completed as of the last submitted annual summary, therefore, there are no new findings to present with respect to this Aim. To summarize our findings briefly, we were able to address whether SPEC1 and SPEC1- SPEC1 and SPEC1-β proteins affect actin organization and cell shape. In NIH-3T3 fibroblasts we have found that SPEC1 induces the formation of plasma membrane blebbing that was not associated with apoptosis. Furthermore, we have shown that SPEC1 expression alters Cdc42 activity in both Cos1 cells and in NIH-3T3 fibroblasts and that an intact CRIB domain is required for this effect. These findings have led to the publication of a peer reviewed journal article entitled, "SPECS, Small Binding Proteins for Cdc42" J. Biol. Chem. (2000) 275:22650-22656.

Specific Aim 2: We will examine the effect of SPEC1 and SPEC1-β on kinase signaling pathways and transcriptional activation.

Summary of Specific Aim 2:
The majority of the work on Specific Aim 2 was completed as of the last submitted annual summary. Briefly, we reported data on the ability of the SPEC1 and SPEC1-β constructs to affect c-Jun N-terminal kinase (JNK activity) using *in vitro* kinase assays. In these experiments we have determined that SPEC1 expression does not stimulate JNK activity on its own and when co-expressed with Cdc42, SPEC1 significantly reduced Cdc42-induced JNK activation. This ability of SPEC1 to downregulate JNK activity required both an intact CRIB domain and C-terminus, as mutations in these regions were less effective and blocking Cdc42-induced JNK activation. These experiments were reported in the journal article entitled, "SPECS, Small Binding Proteins for Cdc42" J. Biol. Chem. (2000) 275:22650-22656.

In addition to investigating the effect of SPEC1 and SPEC1-β on kinase activation, we also proposed to investigate whether SPEC1 or SPEC1-β plays a role in transcriptional signaling. Using luciferase reporter constructs we investigated whether SPEC1 and SPEC1-β expression altered signaling to the TPA responsive element (TRE). Previous work in the field has demonstrated that Cdc42 can lead to TRE activation (Osada et al., 1997), thus it is of interest to determine whether downstream signaling partners such as SPEC1 and SPEC1-β may also play a role in this pathway. Because AP-1 is a family of homo- and heterodimeric transcription factors that bind to the TRE, we investigated whether SPEC1 and SPEC1-β would affect transcriptional activity from an AP-1 luciferase reporter construct. In these studies we found that SPEC1 and SPEC1-β slightly decreased the ability of Cos-1 cells to stimulate AP-1 activity when co-transfected with and AP-1 luciferase reporter construct as compared to an empty vector control (Figure 1). These results were subtle and may be due to non-specific inhibition. Furthermore, because there were no differences between SPEC1 and SPEC1-β on AP-1 activity, we conclude that SPEC1 and SPEC1-β do not appreciably affect transcription from the TPA-responsive element. These data along with data that was previously reported in the last annual summary complete the tasks originally set forth in Specific Aim2.

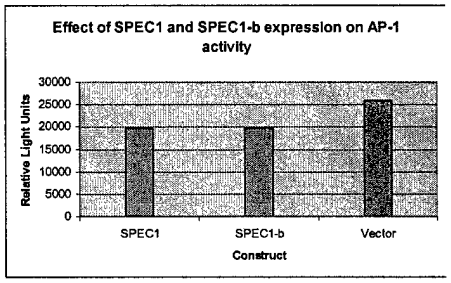

Figure1: SPEC1 and SPEC1-β do not affect AP-1 transcriptional activity.

Specific Aim 3: We will test the hypothesis that the levels of SPEC1 and SPEC1-β proteins in different breast cancer cell lines correlate with breast cancer aggressiveness.

Summary of Specific Aim 3: Preliminary RT-PCR experiments reported in the original research proposal suggested that there were differences in mRNA expression levels between SPEC1 and SPEC1-β and that these may correlate with metastatic aggressiveness. In particular, the SPEC1-β isoform was only expressed in the more highly aggressive MDA-231 and MDA-435 breast cancer cell lines as compared to the less metastatic MCF-7 and T47D breast cancer cell lines. As a correlate to these studies we proposed to generate polyclonal antibodies to the SPEC1 and SPEC1-β proteins and to assess their expression profiles in a panel of breast cancer cell lines. In order to generate polyclonal antibodies to SPEC1 and SPEC1-β proteins we synthesized peptides corresponding to their unique C-termini. The peptides were produced as a cysteine conjugated peptides (Research Genetics) for ease in making subsequent KLH conjugates and peptide affinity columns. After conjugation with KLH, the peptide-KLH conjugate were sent to Rockland, Inc. for immunization into two different rabbits. The test bleeds resulting from these immunizations were affinity purified using peptide affinity columns. Unfortunately, the SPEC1-β immunizations did not yield specific antibodies, so only the SPEC1 antibody was characterized and used for Western blotting experiments.

In order to determine whether the affinity purified serum contained antibodies that were immunoreactive to SPEC1, MCF-7 cells were lysed in SDS-PAGE sample buffer and run on a 4-20% Tris-glysine gel. After blotting to nitrocellulose, the membrane was probed with the SPEC1 rabbit polyclonal antibody, followed by an anti-rabbit-HRP conjugated secondary antibody. In this experiment, MCF-7 whole cell lysates showed an 8 kDa molecular weight species that strongly interacted with the SPEC1 rabbit polyclonal antibody (Figure 2). Also shown is recombinant GST-SPEC1, which was also detectable with the affinity purified SPEC1 antibody (Figure 2). To rule out the possibility that the 8 kDa molecular weight species was non-specific cross reactivity with the secondary antibody, we probed a duplicate blot with anti-rabbit-HRP conjugated secondary antibody alone, but observed no cross-reactivity at the 8 kDa molecular weight (data not shown). These results show that the SPEC1 rabbit polyclonal antibody can detect both endogenous SPEC1 protein from mammalian cell lysates as well as a recombinant GST-SPEC1 fusion protein produced in bacteria.

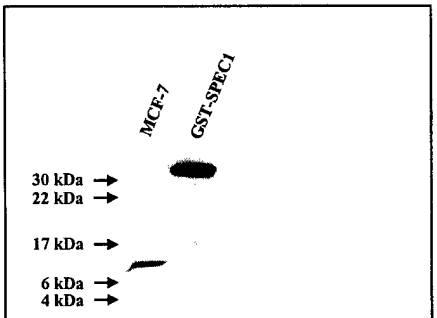

Figure 2: The SPEC1 rabbit polyclonal antibody detects both endogenous and recombinant GST-SPEC1 protein.

Similar Western blotting experiments using HeLa whole cell lysates revealed that endogenous SPEC1 was not detectable in these lysates (data not shown). Based on the hypothesis that HeLa cells contain only very low levels of the SPEC1 protein, we wanted to determine whether transfection of a mammalian expression vector containing the SPEC1 cDNA (pcDNA3-SPEC1) could yield an increase in SPEC1 protein expression. HeLa cells were transfected with a mammalian expression vector containing the SPEC1 cDNA. Twenty-four hours after transfection, the HeLa cells were lysed in SDS-PAGE sample buffer and western blotting was performed using the SPEC1 rabbit polyclonal antibody. As a control, untransfected HeLa cell lysates were also analyzed on the same blot. Here, we were able to detect the SPEC1 protein only in the HeLa cells that were transfected with the SPEC1 cDNA (Figure 3). These results provide further support that the SPEC1 rabbit polyclonal antibody interacts specifically with the SPEC1 protein and that SPEC1 protein expression can be increased by transfection with SPEC1 DNA.

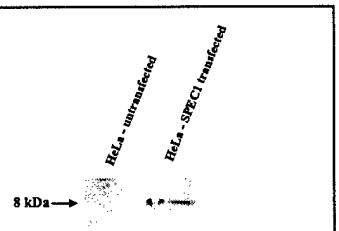

Figure 3: SPEC1 is detectable in transfected HeLa lysates.

The SPEC1 polyclonal antibody was also tested for its ability to detect endogenous SPEC1 protein by immunofluorescence. In these experiments both MCF-7 and HeLa cells were plated onto glass coverslips. After attachment, the cells were fixed, permeabilized, and stained using the affinity purified SPEC1 polyclonal antibody. A vesicular staining pattern was observed in both MCF-7 and HeLa cells (data not shown). This pattern of staining was able to be competed with a pre-incubation with the SPEC1 peptide indicating that the vesicular staining was specific for a SPEC1-like epitope (data not shown). We cannot, however, rule out the possibility that this vesicular staining pattern is due to cross-reactivity with a similar epitope on another protein. The observed vesicular staining pattern was not altered by co-expression of Cdc42, or by treatment with bradykinin, which suggests that the vesicles may not be SPEC1 (data not shown). If the vesicles did represent endogenous SPEC1 staining, then activation of Cdc42 might be expected to cause SPEC1 redistribution from the vesicular compartment, which it does not.

In order to determine if overexpression of SPEC1 was detectable by immunofluorescence using the SPEC1 polyclonal antibody, we transfected HeLa cells with a mammalian expression vector containing the SPEC1 cDNA. Twenty-four hours post-transfection the cells were fixed and processed for immunofluorescence using the SPEC1 antibody. HeLa cells overexpressing SPEC1 showed a cytoplasmic and membrane staining pattern (Figure 4). This membrane localization of SPEC1 might be expected based on its ability to interact with Cdc42. Together, these data indicate that the SPEC1 polyclonal antibody does not detect endogenous SPEC1 protein by immunofluorescence, but does detect overexpressed SPEC1 protein.

Figure 4: Overexpressed SPEC1 protein shows both cytoplasmic and membrane localization.

After characterizing the SPEC protein, we utilized it in Western blotting experiments against cell lysates from a panel of breast cancer cell lines of varying metastatic aggressiveness and ER status. The cell lines represented were placed in order of increasing metastatic aggressiveness and decreasing ER status (Figure 5). Also included in this panel were three ovarian cancer cell lines to the far right. Equal amounts of protein were loaded into each lane. These western blot data indicate that SPEC1 protein levels vary between different breast cancer cell lines, but are not correlated with levels of metastatic aggressiveness or ER status. For instance MCF-7 and T47D breast cancer cell lines are similar in their low level of metastatic aggressiveness and their ER positive status, however, these cells have dramatically different levels of SPEC1 protein, with MCF-7 having a very high amount of SPEC1, while SPEC1 was undetectable in T47D cells. From these data, we conclude that SPEC1 protein levels are not correlated with levels of metastatic aggressiveness in a panel of human breast cancer cell lines. These data along with data that was previously reported in the last annual summary complete the tasks originally set forth in Specific Aim 3.

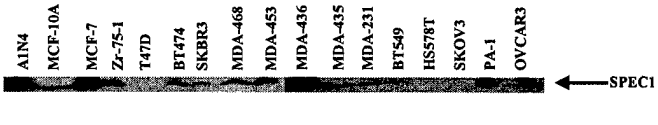

Figure 5: SPEC1 protein levels are not correlated with metastatic aggressiveness.

Additional work completed outside the scope of original Specific Aims:

Because the Specific Aims proposed in the original grant were completed before the length of the funding period, we were able to investigate several other aspects of SPEC1 biology, one of which I would like to report in this annual summary. One area of SPEC1 biology that was particularly interesting to our laboratory was the regulatory mechanisms controlling SPEC1 function. Although SPEC1 contains a centrally located CRIB domain involved in Cdc42 binding, the functional significance of other regions of the SPEC1 molecule remain undefined. Interestingly, a basic amino acid sequence preceding the CRIB domain of SPEC1 resembles a basic stretch of amino acid residues preceding the CRIB domain in N-WASP that is involved in PIP2 binding (Higgs

and Pollard, 1999; Prehoda et al., 2000). This region in SPEC1 consists of nine amino acid residues, K-K-K-R-R-R-I-D-R, that is similar to basic amino acid sequences sequences found in other known phospholipid binding proteins including gelsolin, villin and ezrin.

A fat western filter-binding assay was used to determine whether SPEC1 could bind phospholipids (Stevenson et al., 1998; Dowler et al., 1999). In this assay, nitrocellulose membranes spotted with 100 pmol of 12 different phospholipids (Echelon Research Lab) were incubated with GST-SPEC1, GST-SPEC1-del1 (amino acids 2-27), or GST recombinant proteins. After membrane washing the bound recombinant proteins were detected using GST antibodies followed by ECL development. GST-SPEC1 strongly bound phosphatidylinositol (PtdIns)-3-P, PtdIns-4-P, PtdIns-5-P, phosphatidic acid, and PtdIns-(3,5)P_2, weakly bound PtdIns-(3,4)P_2, PtdIns-(4,5)P_2 and PtdIns-(3,4,5)P_3 but did not bind phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphaditylinositol (Figure 6). A similar pattern of reactivity to that seen with GST-SPEC1 was also observed with 6X-His-tagged SPEC1 (data not shown), indicating that neither the GST nor the 6X His tag interfered with SPEC1-phospholipid binding. To determine whether the basic region of SPEC1 was responsible for PIP binding we utilized a deletion mutant of SPEC1 containing the N-terminus of the protein (amino acids 1-29). This short peptide containing the basic region of SPEC1 showed the same profile as the full length SPEC1 protein confirming that the basic region was likely required for binding the different phospholipids (Figure 15). GST alone was found to only weakly interact with a subset of the phospholipids but this is likely to be non-specific binding due to the high concentrations of protein that were used in the experiments. Together this experimental data supports the possibility that SPEC1 binds different phospholipids than and these phospholipids, like with N-WASP, may control either the activity or the localization of the SPEC1 protein.

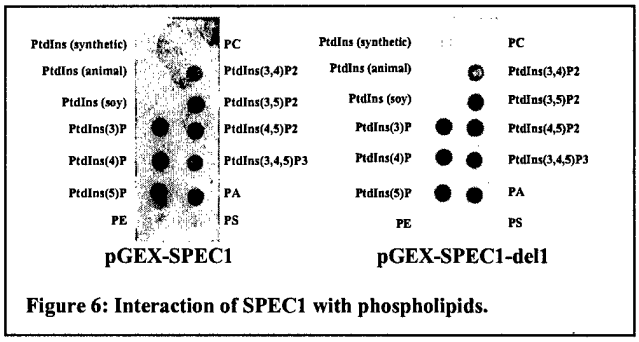

Figure 6: Interaction of SPEC1 with phospholipids.

An analysis of the relative strengths of these interactions showed that SPEC1 interacts most strongly with the PtdIns-monophosphates (Figure 7). Thus, in these experiments we have determined that SPEC1 strongly interacts with a number of phospholipids, including PtdIns-(3)P, PtdIns-(4)P, PtdIns-(5)P, phosphatidic acid and PtdIns-(3,5)P_2. Furthermore, SPEC1 interacted more weakly with PtdIns-(3,4)P_2, PtdIns-(4,5)P_2, and PtdIns-(3,4,5)P_3. An analysis of the relative strengths of these interactions showed that SPEC1 interacts most strongly with the PtdIns-monophosphates. Previously, the non-kinase Cdc42 effector N-WASP was shown to interact with PtdIns-(4,5)-P_2 (Rohatgi et al., 1999; Prehoda et al., 2000). Based on the ability of SPEC1 to bind to a variety of different phospholipids, we also tested whether N-WASP would bind to the same phospholipids as SPEC1. These studies indicate that N-WASP does, in fact, share the same phospholipid binding profile as SPEC1, indicating that N-WASP potentially binds many more phospholipids than previously known (Pirone, Kisailus, Burbelo unpublished observations). These data provide support for the idea that SPEC1 and N-WASP proteins may be regulated by similar mechanisms.

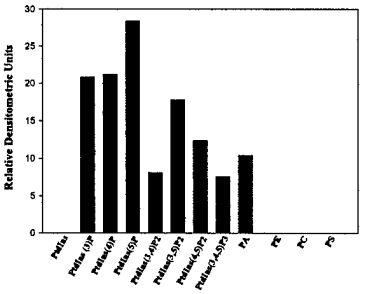

Figure 7: SPEC1 interacts strongly with the PTDIns-monophosphates.

Conclusions

We have completed the work set forth in the original specific aims of this research proposal. First, we have addressed whether SPEC1 and SPEC1-β proteins affect actin organization and cell shape. From these studies, we have determined that in NIH-3T3 fibroblasts, SPEC1 induces the formation of plasma membrane blebbing

that was not associated with apoptosis. Mechanistically, membrane blebs occur at sites where the corical actin is locally depolymerized or detached from the membrane (Cunningham, 1995; Keller and Eggli, 1998, Cunningham et al., 1992) via alteration in cortical actin-binding proteins (Keller and Eggli, 1998), myosin light chain kinase activity (Mills et al., 1998; Huot et al., 1998) and/or focal complex assembly (Huot et al., 1998). It is likely that SPECs function as classical Cdc42 effector proteins by altering the normal signaling pathways leading to actin, myosin, and/or focal complex assembly. Furthermore, we have shown that SPEC1 expression alters Cdc42 activity in both Cos1 cells and in NIH-3T3 fibroblasts and that an intact CRIB domain is required for this effect. SPEC1 also affects the JNK signaling pathway, where SPEC overexpression significantly reduced Cdc42-induced JNK activation. This ability of SPEC1 to downregulate JNK activity required both an intact CRIB domain and C-terminus, as mutations in these regions were less effective and blocking Cdc42-induced JNK activation. Together, these results show that SPECs are capable of modifying Cdc42-dependent signaling at both the biochemical and cellular levels.

We have also examined the mRNA distribution pattern of SPEC1 and SPEC1-β. Using Northern blot analysis, we have determined that the SPEC1 mRNA is ubiquitously expressed and is present in multiple molecular weight species of 1.3, 3.3, 6.3, and 10.2 kb, suggesting complex alternative splicing. For at least two of the molecular weight species, the SPEC1 mRNA transcripts differ in their 3'-untranslated regions. Although the functional consequences of these different 3'-end mRNAs are not known, they may show altered stability or cellular localization. In addition to the alternative splicing in the 3'-end of the SPEC1 mRNA, there is also a SPEC1 splice variant from within the SPEC1 coding sequence, SPEC1-β. SPEC1-β, was detected by RT-PCR as a minor transcript in a variety of human tissues. Interestingly, the mechanism by which the SPEC1-β mRNA transcript is produced involves intron retention. Intron retention occurs in other genes and frequently occurs in cancer. For instance, there is an aberrant inclusion of intron 9 in CD44 transcripts in tumor tissues, including bladder cancer, breast cancer, ovarian cancer, and gastrointestinal tumors (Matsumura et al., 1995; Yoshida et al., 1995; Bolodeoku et al., 1996). Although SPEC1-β is present at low levels in a variety of normal tissues, it is possible that this isoform may occur more frequently in tumor tissues. Experiments using *in vitro* translation confirmed that the SPEC1-β cDNA generates the expected 3.8 kDa protein product. Since the SPEC1-β protein lacks a CRIB domain involved in Cdc42 binding and contains a potential membrane targeting sequence, it may function as a signaling molecule independent of Cdc42 control. Taken together, these results suggest the possibility that the SPEC1-β protein may exist *in vivo* and may have its own unique biological activities. An additional potentially exciting result is that SPEC1 mRNA expression appears to correlate with metastatic aggressiveness, where more highly aggressive cell lines apparently lose SPEC1 mRNA expression.

In order to correlate the levels of SPEC1 and SPEC1-β mRNA expression with protein expression, we attempted to generate polyclonal antibodies to the SPEC1 and SPEC1-β proteins. We were able to generate antibodies that detect SPEC1, but unfortunately, we were not able to generate antibodies that recognized SPEC1-β. Using the SPEC1 antibodies, we show that levels of SPEC1 protein expression are variable in a panel of human breast cancer cell lines, but SPEC1 expression does not correlate with metastatic aggressiveness nor ER status in these cell lines.

In addition to the originally proposed Specific Aims, we have also begun to address other interesting aspects of SPEC1 biology. As a research on this project has progressed, we have made several intriguing discoveries that we have started pursuing. One interesting aspect of SPEC1 biology that we have investigated is the potential for the SPEC proteins to interact with phospholipids in a manner similar to other known Cdc42 binding proteins including the WASP family. Using *in vitro* binding assays with recombinant SPEC1 protein, we have shown that SPEC1 strongly bound phosphatidylinositol (PtdIns)-3-P, PtdIns-4-P, PtdIns-5-P, phosphatidic acid, and PtdIns-(3,5)P_2, weakly bound PtdIns –(3,4)P_2, PtdIns-(4,5)P_2 and PtdIns-(3,4,5)P_3, and did not bind phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphaditylinositol. These results are

interesting because they begin to define at a molecular level the potential regulation of the SPEC family of proteins by phospholipids.